Comparative evaluation of phenotypic methods for detection of MBL producing *Pseudomonas aeruginosa* strains isolated from burn wound infections

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Abstract

Introduction: *Pseudomonas aeruginosa* is a notorious nosocomial pathogen with a resistant drug profile and a predominant isolate in burn wound infections. Recently Metallo beta lactamase (MBL) producing isolates have emerged particularly in *P.aeruginosa* leading to failure of therapy with carbapenems with increased mortality and morbidity in burn units.

Objectives: To detect MBL production among the imipenem resistant isolates of *P.aeruginosa* by different phenotypic methods – Modified Hodge test (MHT), Combined disc test (CDT), Imipenem-EDTA Double disc synergy test (DDST) and MBL-E-test.

Materials and Methods: A total of 108 clinical isolates o*f P.aeruginosa* obtained in 189 pus samples collected from different burn wound sites, were subjected to antimicrobial susceptibility testing by modified Kirby-Bauer disc diffusion method. All the imipenem resistant isolates were further tested for MBL-production by MHT, CDT, DDST and MBL-E-test.

Results: Out of 108 isolates of *P.aeruginosa* 26 (24.07%) ware imipenem resistant. Among 26 imipenem resistant isolates 16(61.54%) were detected as MBL producers by MBL-E-test, 15(57.69%) by DDST, 14(53.85%) by CDT and 11(42.31%) by MHT. Considering MBL-E-test as standard, maximum sensitivity was shown with DDST (93.75%) followed by CDT (87.5%) and MHT (68.75%).

Conclusion: A comparative evaluation of MHT, CDT and DDST against MBL-E-test proved DDST to be most effective, with higher sensitivity and specificity. DDST is observed to be an economical feasible alternative compared to MBL-E-test as a regular screening test in MBL detection in critically ill.

Keywords: *Pseudomonas aeruginosa*, Metallo beta lactamases (MBLs), Modified Hodge test (MHT), Combined disc test (CDT), Imipenem -EDTA Double disc synergy test (DDST), MBL-E-test.

Introduction

Open skin wounds after burn injury serve as preferred sites for bacterial colonisation. Bacterial colonisers originate either from the patient's endogenous flora or transferred from exogenous sources via contact with contaminated external environmental surfaces and hands of health care workers. Microorganisms transferred from the nosocomial environment exhibit more resistance to antimicrobial agents.[1]

In the past few decades, *Staphylococcus aureus* and Gram-negative organisms have emerged as the most common etiologic agents of invasive burn wound infection, with *Pseudomonas aeruginosa* as the most frequent isolate. *Pseudomonas aeruginosa* has been recognized as an emerging nosocomial pathogen and shows intrinsic resistance to a variety of antimicrobials, including the Beta lactam group.

Carbapenems, the beta lactams with the broadest spectrum of activity are the drugs of choice for treatment of infections by penicillin or cephalosporin resistant Gram negative bacilli especially in extended spectrum beta lactamase (ESBL) producing Gram negative infections. However, increasing usage of carbapenems over the past few years resulted in resistance to this group of drugs due to production of carbapenemases (carbapenem hydrolysing enzymes).

Carbapenemases may be defined as beta-latamases that significantly hydrolyze all beta lactams, including carbapenems (at least imipenem or meropenem), with the exception of aztreonam. Carbapenemases involved in acquired resistance are of Ambler molecular classes A, B and D. Class B or the metallo-enzymes (MBL) are the most significant carbapenemases, requiring divalent cations as cofactors for enzymatic activity.[2] Over the last decade MBL producing isolates have emerged particularly in *Pseudomonas aeruginosa*. These isolates have been responsible for serious infections such as septicemia and pneumonia and have been associated with failure of therapy with carbapenems.

Advances in infection control measures in modern burn units in developed countries have played a significant role in decreasing the overall fatality rates from burn wound infection and sepsis. This is not the case in developing countries like India where overcrowding, lack of awareness, poor implementation of safety precautions, lack of medical facilities, low economic status associated with poor compliance of infection control program, injudicious use of broad spectrum antibiotics make the emergence of multi drug resistant organisms a major public health concern.

As there was no data in this area regarding the prevalence of infection with "MBL producing *P.aeruginosa"* and screening protocols for MBL detection, the present study was undertaken to detect MBLs in strains of *Pseudomonas aeruginosa* isolated

from hospitalised burn patients, using phenotypic methods at Department of Microbiology, Siddhartha Medical College, Vijayawada.

Materials and Methods

The present study was conducted over a period of one year, from July 2011 to July 2012 in the Department of Microbiology, Siddhartha Medical College, Vijayawada, Andhra Pradesh. This is a Prospective study and the study material comprises 108 clinical isolates of *Pseudomonas aeruginosa* obtained in 189 pus samples collected from different infected wound sites of 178 patients admitted to the burns ward at Govt. General Hospital,Vijayawada.

Inclusion Criteria

1. Burn patients of all age groups and both sexes.
2. Patients with infected burn wounds with more than seven days of hospital stay.
3. Swabs collected from surface and margins of infected burn wounds from different body sites in burn patients.

Exclusion Criteria

1. Patients on broad spectrum antibiotics / imipenem.
2. Blood, urine and wound biopsy specimens from burn patients.
3. Immunocompromised (HIV positive, those on prolonged treatment with steroids) and cancer patients.
4. Swabs from genital areas.

Specimen Collection, Transport and Processing: All the pus samples were collected from different sites of patients with infected burn wounds following strict aseptic precautions. Two sterile cotton swabs moistened with sterile saline were used for sample collection, which was done after removal of dressing and local wound toilet and immediately transported in a sterile test tube to the Microbiology department. Out of two swabs collected from each site, the first was used to prepare smears for direct microscopic examination.The second swab was inoculated on to nutrient agar, blood agar, Mac Conckey agar and *Pseudomonas* isolation agar. After inoculation swab was put in glucose broth and incubated along with inoculated plates for subculture at 37^{o}C overnight. Isolates were identified according to the standard identification procedures.[3]

Identification of the Isolates: *Pseudomonas aeruginosa* strains were identified by production of bluish green pigment, non lactose fermenting (NLF) colonies on Mac Conckey agar, motile Gram negative bacilli, positive oxidase and catalase tests, oxidative reaction in the O/F medium, Arginine hydrolysis (Moeller decarboxylase method), presence of growth on incubation at 42^{o}C. All the strains were stored at 4°C in stock vials containing semisolid agar medium for the study. At the time of testing, the stock vials were subcultured onto blood agar to check for purity and viability.

The antibiotic susceptibility tests of the *P.aeruginosa* isolates were done by the modified Kirby-Bauer disk diffusion method following CLSI guidelines (Clinical and Lab Standards Institute (CLSI), 2006). Antibiotic disks used for this study were – Imipenem (10µg), piperacillin/tazobactam (100/10µg), amikacin (30µg), ceftazidime (30µg), ciprofloxacin (5µg), colistin (10µg), cefepime (30µg), ceftriaxone (30µg). *P. aeruginosa* ATCC 27853 was used as control. Isolates were considered to be imipenem resistant when the zone around imipenem was = 13 mm, intermediate 14-15 mm and sensitive =16 mm.

All the imipenem resistant strains of *P.aeruginosa* were subjected to different methods of MBL detection namely modified Hodge test, imipenem-EDTA combined disc test, Double disk synergy test and MBL E test.

Tests for Metallo β-lactamase Detection

Modified Hodge Test (MHT): Modified Hodge test was done although not recommended for MBL detection (MHT has been originally described by the Centre for Disease Control for Carbapenemases detection in Enterobacteriaceae). ATCC *E.coli* 25922 at turbidity equivalent to that of 0.5 Mc Farland was inoculated on to the MHA plate. A 10 µg imipenem disk was placed in the centre of the plate. The test strain was heavily streaked from the edge of the imipenem disk to the periphery in four different directions. The plates were incubated at 37°C overnight; zone around the imipenem disk with clover leaf indent is taken as positive test.[4]

Combined Disk Test (Disc potentiation test or Disc enhancement test)

Preparation of Ethylenediaminetetraacetic Acid (EDTA) Stock Solution: 0.5 M EDTA solution was prepared by dissolving 186.1g of disodium EDTA.2H2O in 1000 ml of distilled water and its pH was adjusted to 8.0 by using NaOH. The mixture was then sterilized by autoclaving. EDTA sol (4 µl) was poured on imipenem disks to obtain a desired concentration of 750 µg per disk. The EDTA impregnated antibiotic disks were dried immediately in an incubator and stored at -20°C in airtight vials without desiccant until used.

An overnight broth culture of test strain (opacity adjusted to 0.5 Mc Farland opacity standards) was inoculated on a plate of Mueller Hinton agar. One 10 µg imipenem disk was placed on the agar plate. EDTA impregnated imipenem disk was also placed on same agar plate. The plate was incubated at 37°C for 16 to 18 h. An increase in the zone size of at least 7 mm around the imipenem-EDTA disk compared to imipenem without EDTA was recorded as an MBL producing strain.[5]

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 6-mm blank filter paper disk (Whatman filter paper no.1) which contained approximately 750 µg of EDTA. A 10 µg 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 (750 microgram). After overnight incubation, the presence of an enlarged zone of inhibition towards the EDTA disk was interpreted positive for an MBL producer.[4]

E-Test: E-test metallo-beta-lactamase strips consist 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 Mc Farland'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 was interpreted as being suggestive of MBL production.[6]

Results

Out of 189 pus samples processed, 108 (57.14%) were culture positive for *P.aeruginosa*. All 108 *P.aeruginosa* strains were subjected to anti microbial susceptibility testing by Kirby Bauer disc diffusion method (Table-1). Most of the isolates were resistant to ceftriaxone (87.96%) followed by cefepime (80.56%), gentamicin (67.59%), ceftazidime (66.67%), ciprofloxacin (61.11%) and piperacillin + tazobactum (50.92%). The isolates showed highest susceptibility to imipenem (75.92%) followed by moderate activity with amikacin (51.86%), piperacillin + tazobactum (49.08%).

Table 1: Antibiogram of *P.aeruginosa* isolates -Kirby bauer disk diffusion method (n=108)

S.No.	Antibiotics	Sensitive isolates (No & %)	Resistant isolates (No & %)
1	Ceftriaxone	I3 (12.04%)	95 (87.96%)
2	Cefepime	21 (19.44%)	87 (80.56%)
3	Ceftazidime	35 (32.41%)	73 (67.59%)
4	Gentamicin	36 (33.33%)	72 (66.67%)
5	Ciprofloxacin	42 (38.89%)	66 (61.11%)
6	Piperacillin + Tazobactum	53 (49.08%)	55 (50.92%)
7	Amikacin	56 (51.86%)	52 (48.14%)
8	Imipenem	82(75.92%)	26(24.07%)

Among108 isolates of *P.aeruginosa*, 26(24.07%) were imipenem resistant (Kirby Bauer method) and 16 (14.81%) isolates were MBL producers (as detected by various phenotypic methods namely modified Hodge test, Combined disc test, Double disk synergy test & MBL E-test). Majority (61.53%) of imipenem resistant isolates were MBL producers (16 of 26 isolates) [Table 2].

Table 2: Imipenem resistance & MBL production in *P.aeruginosa* strains (n=108)

Total no. of isolates	Imipenem resistant strains (No. & %)	
108	26 (24.07%)	
	MBL producers (No. & %)	MBL non producers (No. & %)
	16(14.81%)	10(9.26%)

Out of 26 imipenem resistant isolates, 16 (61.54%) were detected as MBL producers by MBL E-test, 15 (57.69%) by DDST, 14 (53.85%) by CDT and 11 (42.31%) by MHT (Table 3).

Table 3: **MBL detection - Comparison by different methods (n=26)**

S.No.	Method	MBL positive isolates (No & %)
1	Modified Hodge test (MHT)	11(42.31%)
2	Combined disc test (CDT)	14(53.85%)
3	Double disk synergy test (DDST)	15(57.69%)
4	MBL E-test (MIC test)	16(61.54%)

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive + False negative}} \text{ X } 100$$

True positive- positive result obtained in both the reference method and the screening method.

False negative – positive result obtained in reference method but negative result obtained by screening method. Considering MBL E test as standard for MBL detection, maximum sensitivity was shown with DDST (93.75%) followed by CDT (87.5%) and MHT (68.75%) [Table 4].

$$\text{Specificity} = \frac{\text{True negative}}{\text{True negative + False positive}} \text{ X } 100$$

True negative- negative result obtained in both the reference method and the screening method.

False positive- negative results obtained in the reference method but positive result obtained in the screening method. All the three tests were found 100% specific for MBL detection (Table 4).

Table 4: Efficacy correlation
Sensitivity and Specificity gradient with "MBL E test" as standard

S.No.	Method	True positive	True negative	False negative	False positive	Sensitivity	Specificity
1.	MHT	11	10	16-11=5	0	68.75%	100%
2.	CDT	14	10	16-14=2	0	87.5%	100%
3.	DDST	15	10	16-15=1	0	93.75%	100%

$$\text{PPV} = \frac{\text{True positive}}{\text{True positive + False positive}} \text{ X } 100$$

PPV or precision rate is the proportion of positive test results that are true positives.

All the three methods showed PPV of 100% indicating their precision as a diagnostic test in detecting true positives (Table 5).

$$\text{NPV} = \frac{\text{True negative}}{\text{True negative + False negative}} \text{ X } 100$$

NPV is a summary statistic used to describe the performance of a diagnostic testing procedure and defined as the proportion of subjects with a negative test result who are correctly diagnosed. A high NPV for a given test means that it is more likely to be correct in its assessment. Highest NPV of 90.90% was shown with DDST followed by 83.3% with CDT and 66.67% with MHT (Table 5).

Table 5: Efficacy correlation
PPV* and NPV gradient with "MBL E Test" as standard**

S.No.	Method/Test	True positive	False positive	True negative	False negative	NPV	PPV
1.	MHT	11	0	10	5	66.67%	100%
2.	CDT	14	0	10	2	83.3%	100%
3.	DDST	15	0	10	1	90.90%	100%

***Positive predictive value. **Negative predictive value**

All MBL producers were 100% resistant to piperacillin + tazobactum, ceftazidime, ceftriaxone, cefepime and ciprofloxacin which is not the case with MBL non producers. Also MBL producers were more resistant to aminoglycosides and flouroquinolones compared to MBL non producers. All MBL producers were multidrug resistant but are colistin sensitive (Table 6).

Table 6: MBL producers Vs MBL non producers (n=26) (A comparative Antibiogram)

S.No.	Antibiotics	Sensitive MBL producers (No & %) (n=16)	Sensitive MBL non producers (No & %) (n=10)
1	Ceftriaxone	0(0%)	1(10%)
2	Cefepime	0(0%)	2(20%)
3	Ceftazidime	0(0%)	3(30%)
4	Ciprofloxacin	0(0%)	5(50%)
5	Piperacillin + tazobactum	0(0%)	6(60%)
6	Gentamicin	1(6.25%)	5(50%)
7	Amikacin	1(6.25%)	7(70%)
8	Colistin	16(100%)	10(100%)

Discussion

A total of 108 *P. aeruginosa strains* were recovered from 189 pus samples obtained from burn wounds.

Higher culture positivity (57.14%) for *P. aeruginosa* was observed in samples from infected burn wounds in the present study, indicating *P. aeruginosa* as a common isolate in burns ward of our setup. Prevalence of *Pseudomonas* species in the burns ward may be due to the fact that organism thrives in a moist environment.

High isolation rate of *P. aeruginosa* in burn wound infections, in present study is consistent with others' studies by Rajput et al.,2008 (55%) and Kalantari et al.,2012 (56.8%).[7] Altoparlak, U et al., 2004, also quoted *P. aeruginosa* as the most common cause of burn wound infections in many centers.[8]

In a study of autopsies by Harish Dasari et al., 2008 on cases of death following burns, at mortuary Government Medical College and Hospital, Chandigarh, over a period of two and half years (January 2006 to 31st May 2008), *Pseudomonas aeuroginosa* (31%) and *Klebsiella* (24%) were the most common micro-organisms isolated from the splenic smears.[9]

Table 1 shows antibiogram of *P. aeruginosa* isolates (Kirby – Bauer disc diffusion method).

Most of the isolates were resistant to ceftriaxone (87.96%) followed by cefipime (80.56%), ceftazidime (67.59%), Gentamicin (66.67%), ciprofloxacin (61.11%) and piperacillin with tazobactum (50.92%). Most of the *P. aeruginosa* isolates were susceptible to imipenem (75.92%), with moderate susceptibility to Amikain (51.86%) and piperacillin with tazobactum (49.08%).

This profile indicates that, *P. aeruginosa* isolates prevalent in the burns ward were multidrug resistant to usually used broad spectrum antibiotics like β-lactams, aminoglycosides & fluoroquinolones, probably due to empirical use of broad-spectrum antibiotics and non adherence to hospital antibiotic policy. Judicious selection of antibiotics for empirical therapy based on contemperory sensitivity profile of *P. aeruginosa* may help combat the emergence of multidrug resistant strains.

Among the 108 isolates, 26 (24.07%) were resistant to imipenem. In the present study imipenem resistance in *P. aeruginosa* was found to be 24.07%. This is quite high as compared to other studies among the clinical isolates of P. aeruginosa as reported by Navaneeth et al., 2002 (12%), Hemalatha et al., 2005 (16%) and Agarwal et al., 2008 (8.05%).[10-12] MBL production in present study is in closer agreement with studies by Jazani et al, 2012 (21%), Varaiya et al., 2008 (26%) and Bose et al, 2012 (30%).[13-15] However, a high prevalence of imipenem resistance was reported by Irfan et al, 2008 (59.52) and Bhalerao D S, et al., 2010 (67.5%).[5, 16]

The present study indicates considerable prevalence (24.07%) of imipenem resistant *P. aeruginosa* in the burns ward, necessitating further screening of these isolates for MBL production. Of 26 imipenem resistant strains, 16 (14.81%) were detected as MBL producers by different phenotypic methods (Table-2). The remaining 10 MBL negative imipenem resistant isolates may have other mechanisms of resistance such as impermeability of outer membrane or active efflux. Observations in this context were in accordance with other studies reported by Navaneeth et al., 2002 (12%), Hemalatha et al., 2005 (14%) and Bose et al., 2012 (15.71%).[10,11,15] Mihani F et al., 2007 (41%) and Bhalerao et al., 2010 (45%) noted higher prevalence of MBL producers among *P.aeruginosa* isolates compared to the present study.[5,17]

Present study showed higher rate i.e., 61.53% (16/26) of imipenem resistance being mediated through MBLs, which correlated with a study by Bhalerao et al., 2010 (66.7% of MBL mediated resistance). This study indicates the appearance of MBL-producing *P. aeruginosa* at Government General Hospital, Vijayawada. The occurrence of MBL positive isolates poses not only a therapeutic problem but is also a serious concern for infection control management. These facts are to be considered before deciding the antibiotic policy in burn wound management.

Table 3 summarizes the results obtained by different methods employed in this study to detect MBL producing *P. aeruginosa* namely modified Hodge test (MHT), Double disk synergy test (DDST) and MBL E test, which were performed on all 26 imipenem resistant isolates.

Of the 26 imipenem resistant isolates, MHT could pick 11 isolates as MBL positive while CDT, DDST and MBL E-test detected 14 (53.85%), 15 (57.69%) and 16 (61.54%) of MBL producers respectively. In the present study, MBL E-test could detect maximum number of MBL producers (16/26) followed by DDST (15/26).

Tables 4 and 5 represent comparative evaluation of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of three phenotypic methods against MBL E test. DDST showed a higher sensitivity (93.75%) followed by CDT (87.5%) and MHT (68.75%). All the three tests were found 100% specific for MBL detection. Peak PPV (100%) observed with all the three tests; indicate all positives detected by the three tests are true positives (accuracy). The NPV of MHT, CDT & DDST were 66.67%, 83.3% and 90.90% respectively. Higher NPV for DDST indicates that the test was more reliable in detecting true negatives.

Since there are no standard CLSI guidelines for MBL detection, different studies have reported the use of different methods.[15] Most studies have used IPM-

EDTA combined disc, double disc synergy test using IPM-EDTA and modified Hodge test. After the review of literature and experience of contemporary workers, the above four methods (DDST, CDT, MHT, MBL E test) were employed in the present study.[11,15] It was observed in the present study that MBL E test followed by DDST were effective options for MBL detection. However DDST was cost effective.

Table 6 compares antibiogram of MBL and MBL-non producing *P. aeruginosa*.

All MBL producers were 100% resistant to piperacillin+tazobactum, ceftazidime, ceftriaxone and cefepime while MBL-non producers showed a sensitivity 60%, 30%, 10% and 20% respectively for the same antibiotics tested.

Besides exhibiting total resistance to β-lactam antibiotics, MBL producers showed a poorer sensitivity profile to aminoglycosides (6.25%) and absolute resistance to fluoroquinolones. MBL non producers however showed higher sensitivity to amikacin (70%) and moderate sensitivity to ciprofloxacin (50%).

Similar resistance pattern to different group of drugs between MBL producers and non producers were shown in other studies by Bashir et al., 2011 at Kashmir and Bose et al., 2012 at Loni, Maharastrsa.[6,15] All MBL & MBL-non producers were 100% colistin sensitive. Carriage of both the genes coding for MBLs and antibiotic resistant determinants to other classes by the same plasmid could be the reason for the above observations.

In view of resistance to broad spectrum antibiotics in *P. aeruginosa*, consequent to MBL production, reintroduction of toxic polymixin group of drugs (polymixin B and colistin) which were earlier in vogue became inevitable presently.

Conclusion

In burn wound injuries, infection by MBL producing *P.aeruginosa* is on the rise, critically influencing the clinical outcome and health care costs. Among four phenotypic tests employed for MBL detection (MBL E test, modified Hodge test, combined disk test and double disk synergy test) MBL E test is most effective but economic feasibility makes DDST a preferable alternative for screening.

All microbiological laboratories associated with the management of burn wound infection must be suitably equipped with an effective screening protocol for prompt detection of MBL producers and to help the clinician regarding the choice of appropriate antibiotics. Prompt isolation of MBL positive *P.aeruginosa* infected burn wound patients from the rest, barrier nursing, strict observance of universal precautions goes a long way in better management of burn wound infections. While framing antibiotic policy, in particular for serious burn wound infections by multidrug resistant *P.aeruginosa*, the rise in MBL production, amongst the *Pseudomonas* must be carefully considered.

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