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Comparison of Various Phenotypic Methods for the Detection of Metallo-beta-lactamases in *Pseudomonas aeruginosa*

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Authors' contributions

This work was carried out in collaboration between all authors. Author SR designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors NB and MM managed the analyses of the study. Author SF managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To evaluate the screening antibiotic and phenotypic test that can be used to confirm metallo-beta-lactamase (MBL) production in clinical isolates of *Pseudomonas aeruginosa* and to find out the prevalent MBL gene in them.
Materials and Methods: Three hundred and six isolates of *P. aeruginosa* were screened for resistance to meropenem (MEM), ceftazidime (CAZ) and imipenem (IMP). Isolates resistant to any of these were taken as screen test positive for MBL production and subjected to double disc synergy test (DDST) and combined disc synergy test (CDST), using MEM, CAZ and IMP with and without EDTA. Broth microdilution with MEM and IMP with and without EDTA was done to confirm

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MBL production (four fold reduction in minimum inhibitory concentration, MIC). Polymerase chain reaction (PCR) for bla_{VIM} and bla_{IMP} was done to find the prevalent gene in *P. aeruginosa* isolates.
Results: MEM picked up the highest number of MBL positive isolates 28.8% (n=76). CDST using MEM confirmed all the 76 screen test positive isolates to be MBL producers. Sensitivity of CDST using MEM, CAZ and IMP was 100%, 92.7% and 88.4% respectively. MIC by microbroth dilution for MEM and IMP was done for 76 MEM and 70 IMP positive isolates. For MEM maximum number of isolates had an MIC of 16 µg/ml and for IMP maximum number of isolates had an MIC of 32 µg/ml. bla_{VIM} was the predominant MBL gene in *P. aeruginosa* isolates.
Conclusion: Meropenem was found to be a better screening as well as confirmatory agent for MBL detection. bla_{VIM} was the predominant MBL gene in *P. aeruginosa* in our hospital.

Keywords: Combined disc synergy test; double disc synergy test; Metallo-beta-lactamase; polymerase chain reaction.

1. INTRODUCTION

Pseudomonas aeruginosa emerged as a major human pathogen in the 1960's because of its ability to cause infections in immunocompromised individuals, burn patients and Cystic Fibrosis patients. Since that time the organism has become one of the most serious causes of nosocomial bacterial infections notable in the lung, blood and urinary tract [1]. Antibiotic resistance in *P. aeruginosa* is interplay of many mechanisms, the most notable among them being production of carbapenemases. Among the carbapenemases, metallo-β-lactamases (MBL's) are predominately produced by *P. aeruginosa.* These enzymes belong to Ambler's class B and Bush-Jacoby Mederios Group 3 and hydrolyze all β-lactam agents including carbapenem class of antibiotics [2]. Seven major types of MBL's have been described so far; IMP, VIM, SPM, SIM, GIM, AIM-1 and the most recent NDM-1, with bla_{IMP} and bla_{VIM} being the most common types of MBL's. From India only bla_{VIM} and NDM-1 have been reported in *P. aeruginosa*. Possession of such enzymes along with mutations that increase the levels of a number of efflux systems that pump out β-lactam antibiotics may result in even higher MIC's against these drugs resulting in the inability to use such drugs even if they are only minimally degraded by β-lactamases [3].

The occurrence of MBL producing *P. aeruginosa* in a hospital setting poses a therapeutic problem and is a serious concern for infection control management due to their survival in extremes of environment and rapid spread. In the absence of any clear cut guidelines for the detection and confirmation of MBL production in *P. aeruginosa* from the clinical laboratory standards institute (CLSI) several screening methods are employed by different laboratories. Such methods include double disk synergy tests (DDST), combined disk synergy tests (CDST), the Hodge test, MBL E-test strips and the microdilution methods. Among them the most widely accepted standardized MBL confirmation method is the MBL E-test. However its high cost precludes its routine use. In turn methods like DDST and CDST are widely used due to their low cost and ease of performance however they have shown discordant results depending upon the methodology used, β-lactam substrates, MBL inhibitors and the bacterial genus tested [4-6].

The present study was undertaken to determine which of the antibiotics; meropenem (MEM), imipenem (IMP) and ceftazidime (CAZ) can serve as a good screening as well as confirmatory agent for MBL detection; to evaluate the efficacy of DDST and CDST as a confirmatory test and find out the prevalent MBL gene in clinical isolates of *P. aeruginosa* in this part of the country.

2. MATERIALS AND METHODS

A total of 306 non duplicate isolates of *P. aeruginosa* were isolated from clinical samples (from patients of all age groups) like blood, sputum, urine, pus and other body fluids in the Department of Microbiology; Sher-i-Kashmir Institute of Medical Sciences (SKIMS) during the study period (Aug 2013-July 2014). The study was approved by the institute's ethical committee bearing clearance number SKIMS/ Acad/ 23 of 2013.

2.1 Screening for MBLs

Antibiotic sensitivity testing was performed by the Kirby-Bauer disc diffusion method as per the Clinical Laboratory Standards Institute (CLSI) guidelines [7]. An isolate of *P. aeruginosa* that was resistant to IMP (10 µg) or MEM (10 µg) or

CAZ (30 µg) was considered screen-test positive for MBL and included in the study.

2.2 Confirmation of MBL Production

All screen test positive isolates were subjected to combined disc synergy test (CDST) using IMP, MEM and CAZ disc along with EDTA and double disc synergy test (DDST) using the same discs. The DDST was performed as described by Lee et al. [8] and the CDST was performed as described by Yong D et al. [9] Organisms that showed increased zone of inhibition by 7 mm or more [6] around any or all of the three disc with EDTA or showed increase in 5-28 mm inhibition around only CAZ-EDTA disc [10] as compared to IPM, MEM and CAZ discs alone, respectively, were considered to be MBL producers. A blank disc of EDTA served as control. Enhancement of the zone of inhibition around IMP and/or MEM and/or CAZ towards the EDTA disc in comparison with the zone of inhibition on the far side of the corresponding antibiotic disc was interpreted as a positive result [11,12].

2.3 EDTA Plus MEM/IPM Microdilution Test

Minimum inhibitory concentrations (MIC's) were determined in 96 well micro titre plates using 50 µl of Muller Hinton broth, 5 µl of bacterial inoculum (5 X 10^4 CFU: Soon after preparation) and 50 µl of corresponding concentration of the antibiotic per well as per CLSI guidelines. The concentration of IPM/MEM tested was in the range of 0.25 - 512 µg/ml. Reduction in MIC of IPM and MEM were determined by adding 5-µl mixture of chelator EDTA 0.5 mM to the second row, just before inoculation of wells with broth culture. Growth controls without IPM/MEM but with and without chelator mixtures were also included. Stock solutions for IMP/MEM were prepared in advance from which working solution were prepared at the time of putting up the test. Results were recorded by visual inspection of micro titre plates after 18 hours of incubation at 37℃. The test was considered valid when acceptable growth (more or equal to 2-mm button or definite turbidity) was seen in the positive control well. Absence of turbidity or a button of less than 2 mm diameter in the test well was thus taken as the MIC of the organism under test. A ≥ four-fold IPM and/or MEM MIC reduction in presence of chelators as compared to MIC without them was taken as cutoff value for MBL production.

2.4 PCR for the Detection of MBL Producing Genes *bla*$_{IMP}$ and *bla*$_{VIM}$

PCR was performed for the detection of MBL producing genes *bla*$_{IMP}$ and *bla*$_{VIM}$ in the recovered isolates of *P. areuginosa.* Fresh cultures of the test organism and control strains were suspended in 500 µl of saline and vortexed to get a uniform suspension. Cells were lysed by heating at 100℃ for 10 min and cellular debris removed by centrifugation at 8000 rpm for 5 min. The supernatant was used as a source of template.

PCR was carried out in a 25 µl solution containing a mixture of dNTPs 2.5 µl, 1 µl each of forward and reverse primer, 2.5 µl PCR buffer, 2.5 µl $MgCl_2$, 8.2 µl MiliQ water, 0.3 µl of Taq polymerase and 5 µl of extracted DNA. The primers used were VIM-forward (5'-GTT TGG TCG CAT ATC GCA AC -3') VIM-reverse (5'AAT GCG CAG CAC CAG GAT AG-3'), which amplified a 382-bp amplicon and IMP- forward (5'-GAA GGY GTT TAT GTT CAT AC-3') IMP-reverse (5'-GTA MGT TTC AAG AGT GAT GC-3') which amplified a 587-bp amplicon.

An initial denaturation step, carried out at 94℃ for 2 min was followed by 30 cycles of DNA denaturation at 94℃ for 1 min. Annealing was achieved at 54℃ for 1 min and extension at 72℃ for 15 mins with a holding temperature of 72℃ for 5 mins. PCR products were stored at 4℃. The amplicons were electrophoresed in 1% agarose gel and visualized with UV light after staining with ethidium bromide.

2.5 Statistical Analysis

The antibiotic sensitivity pattern of *P. aeruginosa* MBL+ versus MBL- was compared using Chi-square test and p-values were reported for individual antibiotics. Sensitivity and Positive Predictive Value (PPV) of DDST and CDT in identifying the isolates was also reported. Statistical analysis was done using www.openepi.com. A p-value of <0.05 was considered statistically significant.

3. RESULTS

Out of the 306 isolates of *P. aeruginosa*, 76 (24.8%) were screen test positive for MBL production and 230 (75.2%) were screen test negative when tested with MEM or IMP or CAZ discs. MEM was able to pick up 76 (100%) MBL

positive isolates whereas IMP was able to pick up 70 (92.1%) and CAZ 72 (94.7%).

Significantly higher resistance among MBL positive *P. aeruginosa* isolates was seen to the antibiotics tested; with 54 (71.1%) being resistant to amikacin, 56 (73.7%) being resistant to gentamicin, 50 (65.8%) to tobramycin, 53 (69.7%) to carbenicillin, 59 (77.6%) to ticarcillin + clavulanic acid, 57 (75%) to piperacillin + tazobactam, 65 (85.5%) to ciprofloxacin and 55 (72.4%) to levofloxacin. In addition 72 (94.7%) MBL positive isolates of *P. aeruginosa* were resistant to ceftazidime, 70 (92.1%) to imipenem and 76 (100%) to meropenem. The isolates however displayed uniform sensitivity to Polymyxin B. Comparison of the susceptibility profile of MBL producing and non-producing *P. aeruginosa* isolates is given in Table 1.

Seventy six MEM resistant isolates (that included the ones found resistant to IMP and CAZ as well) were subjected to DDST and CDST using MEM, IMP and CAZ discs along with EDTA. DDST detected MBL production in 74 (97.4%) isolates using MEM disc and EDTA disc, whereas it detected MBL production in 68 (89.5%) and 62 (81.6%) isolates using CAZ and EDTA disc and IMP and EDTA disc respectively. CDST on the other hand confirmed all the 76 (100%) screen test positive isolates to be positive for MBL enzyme using MEM/EDTA disc followed by 70 (92.1%) and 66 (86.8%) isolates by CAZ/EDTA and IMP/EDTA disc respectively. All the isolates that were picked up by DDST using MEM, CAZ and IMP disc along with EDTA disc were found to be MBL positive by CDST Comparison of the results of DDST and CDST and their sensitivity and positive predictive value is given in Table 2, Fig. 1.

MIC by microbroth dilution for 76 MEM and 70 IMP screen test positive isolates done for confirmation of MBL production is shown in Table 3. Four fold reductions in MIC's of both the antibiotics for all the *P. aeruginosa* isolates was seen when combined with EDTA. For MEM, maximum number of isolates had an MIC of 16 µl/ml where as for IMP maximum isolates had an MIC of 32 µl/ml. Polymerase chain reaction

Table 1. Antibiotic susceptibility profile of *P. aeruginosa* isolates

Antibiotic	MBL +ve (n=76)				MBL –ve (n=230)				*P*-value
	S		R		S		R		
	N	%	N	%	N	%	n	%	
Amikacin	22	(28.9)	54	(71.1)	104	45.2	126	54.8	*P*=0.012
Gentamicin	20	(26.3)	56	(73.7)	92	40	138	60	*P*=0.032
Tobramycin	26	(34.2)	50	(65.8)	121	52.6	109	47.4	*P=0.005*
Carbenicillin	23	(30.3)	53	(69.7)	115	50	115	50	*P*=0.003
Ticarcillin + Clavulanic acid	17	(22.4)	59	(77.6)	102	44.4	128	55.6	*P*=0.001
Piperacillin + Tazobactam	19	(25)	57	(75)	136	59.1	94	40.9	*P*<0.0001
Ciprofloxacin	11	(14.5)	65	(85.5)	74	32.2	156	67.8	*P*=0.003
Levofloxacin	21	(27.6)	55	(72.4)	143	62.2	87	37.8	*P*<0.0001
Polymyxin B	76	(100)	0	(0)	230	100	0	0	-

Table 2. Performance of DDST and CDST using different antibiotics

	No of isolates picked (%)	No of isolates missed (%)	Sensitivity	PPV*
Meropenem				
DDST	74 (97.4)	2 (2.6)	97.4%	100%
CDT	76 (100)	0	100%	100%
Imipenem				
DDST	62 (81.6)	14 (18.4)	81.6%	100%
CDT	66 (86.8)	10 (13.2)	86.8%	100%
Ceftazidime				
DDST	68 (89.5)	8 (10.5)	90.5%	100%
CDT	70 (92.1)	6 (7.9)	92.7%	100%

**PPV: positive predictive value*

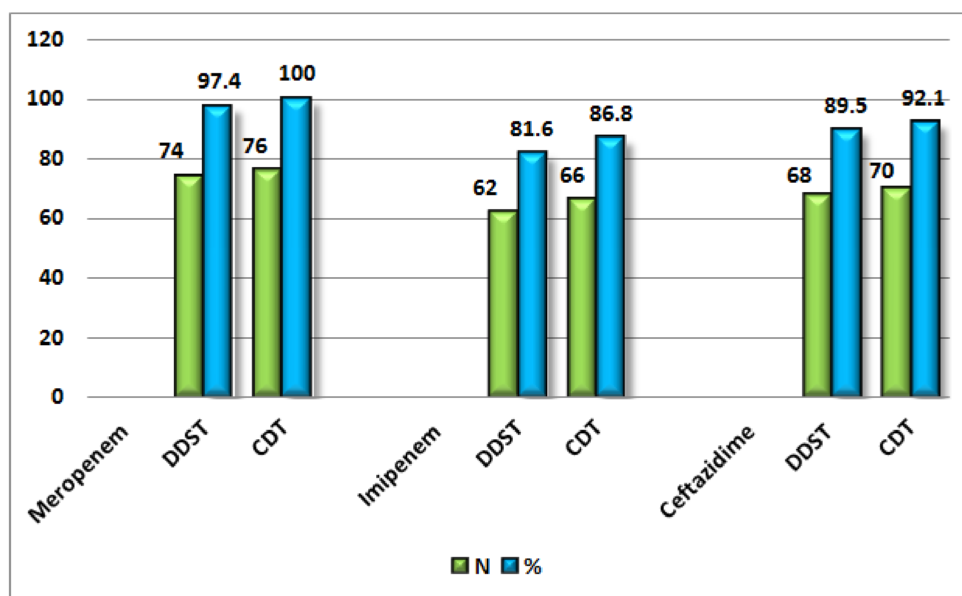

Fig. 1. Performance of DDST and CDST using various antibiotics. Green bar denotes the number of isolates and blue bar denoted the percentage

Table 3. MIC's of screen test positive *P. aeruginosa* isolates (n=76)

MIC (µg/ml)	Meropenem	Meropenem + EDTA	Imipenem	Imipenem + EDTA
0.25	0	0	0	0
0.5	0	7	0	4
1	0	11	0	25
2	0	26	0	11
4	0	18	0	12
8	8	14	3	18
16	30	0	10	0
32	21	0	29	0
64	14	0	20	0
128	3	0	7	0
256	0	0	1	0
512	0	0	0	0

was done for all the 76 *P. aeruginosa* isolates. bla_{VIM} was the predominant MBL gene in all the isolates, with none of the isolates harboring the bla_{IMP} gene (Photo 1).

4. DISCUSSION

A higher number of MBL producing *P. aeruginosa* were seen in our study; 24.8% (76/306) as compared to an earlier study from the same institute that reported 11.7% MBL prevalence in the organism, [13] as well as other studies from across the country [14-18]. Out of the three antibiotics tested, MEM picked up the highest number of MBL positive *P. aeruginosa* isolates as compared to CAZ and IMP. In their study, Buchunde et al. [2] found that MEM and CAZ picked the highest number of MBL positive isolates, 19.3% (63/326) as compared to IMP 17.8% (58/326). Likewise Bansahankari et al. [12] reported that all the MBL producing *P. aeruginosa* were resistant to MEM, 100% with only 93.1% being resistant to IMP. Many authors have recommended the use of CAZ in place of IMP for the screening of MBL producers, especially for the isolates who have high MIC for CAZ [2].

Although MBL positive *P. aeruginosa* isolates were recovered more from male than female

patients, the difference was not significant, however significantly more MBL positive *P. aeruginosa* isolates were recovered from patients in the age group of 40-59 years; *P*=0.017. Bansahankari et al. [12] observed no significant difference in the age distribution pattern of MBL positive and negative isolates with infection being more prevalent in the 40-60 year age group. Anupurba et al. [19] in their study stated that males are more likely to be predisposed to infection due to *P. aeruginosa.* Majority of the MBL positive isolates were recovered from pus, 31.6% (24/76). Our results are in agreement to those reported by other authors. 60,57,56 Also specimens obtained from patients housed in the plastic surgery ward yielded the maximum number of MBL positive *P. aeruginosa isolates* 18.4% (14/76). Significantly higher resistance pattern was seen in the MBL producing *P. aeruginosa* isolates to the antibiotics tested in our study, similar to what was reported by other authors from across the country [20-22].

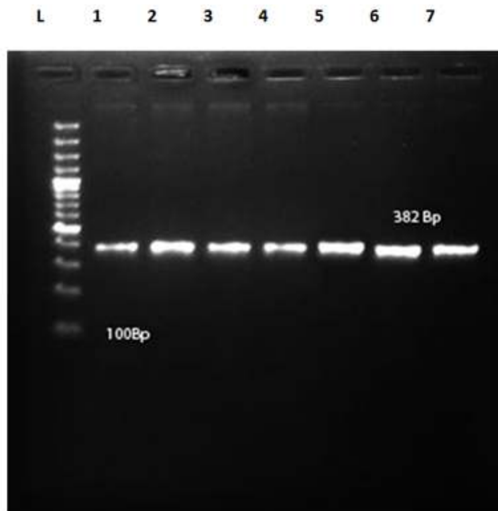

Photo 1. Agarose gel electrophoresis showing positive amplification of 382 base pairs specific for *bla*$_{VIM}$ of *Pseudomonas aeruginosa*. Lane L: Size marker (100-bp DNA ladder); Lane 1-7; test strains showing positive results for *bla*$_{VIM}$

Taking PCR as the gold standard for MBL detection, the sensitivity of DDST and CDST using MEM was 97.4% and 100% respectively. With CAZ the sensitivity was 90.5% for DDST and 92.7% for CDST. Low sensitivity of 84.4% for DDST and 88.4% for CDST was seen with Imipenem. Thus both the phenotypic tests (CDST and DDST) using IMP and CAZ missed confirmation of some isolates found positive by MEM antibiotic using the same technique. Based on these results we found MEM to be a better screening agent and CDST to be a better confirmatory test that can be employed for routine detection of MBL enzyme in *P. aeruginosa* isolates. MEM and CAZ as better screening antibiotics were also seen by Buchunde et al. [2]. However the authors found MEM DDST to perform better as compared to our findings where CDST using MEM was seen to have better sensitivity. Wadekar MD et al. [22] state that the DDST lacks sensitivity because of the problem of optimal disc space and the correct storage of the clavulanic acid containing discs. Moreover the interpretation is subjective.

Mendiratta et al. [17] in their study reported CAZ DDST to be better than IMP DDST; similar results were seen by us. CAZ has been recommended by many authors for screening of MBL producers as CAZ is more stable than IMP. As far as IMP is concerned, CDST using this antibiotic performed better than DDST. Manoharan et al. [23] found CDST using IMP+EDTA (87.8% sensitivity) to be a better predictor of MBL production as compared to MEM+EDTA and CAZ+EDTA. Qu TT et al. [24] reported CDST using IMP+EDTA to be a better screening method for MBL production in *P. aeruginosa* strains. Likewise Sopasri et al. [25] and Behera et al. [21] also found IMP CDST to be better than IMP DDST.

MIC by microbroth dilution for 76 MEM and 70 IMP screen test positive isolates was done for the confirmation of MBL production. The MIC's of both the antibiotics for all the *P. aeruginosa* isolates was in resistant range and showed four fold reductions when combined with EDTA thus confirming all the screen test positive isolates as MBL producers.

The MBL gene in *P. aeruginosa* isolated from our hospital was found to be *bla*$_{VIM}$ with none of the organisms harboring the *bla*$_{IMP}$. Buchunde et al. [2] in their study confirmed 63 screen test positive *P. aeruginosa* isolates to contain *bla*$_{VIM}$. Manoharan et al. [23] also reported only *bla*$_{VIM}$ type MBL from 17 of the 20 E test confirmed MBL positive *P. aeruginosa* isolates from seven different centers of India. Similarly in a study carried out by Castanheira et al. [26] the author's demonstrated only *bla*$_{VIM-2}$ in 53.2% *P. aeruginosa* isolates. Johann et al. [27] reported *bla*$_{VIM}$ in 43% and *bla*$_{IMP}$ in 2% *P. aeruginosa* isolates in their study.

5. CONCLUSION

To conclude, characterization of MBL producing bacteria especially *P. aeruginosa* is of utmost importance as they can thrive even in the most hostile environments. The dissemination of such resistant strains poses a potential threat to the current infection control practices. We recommend routine detection of MBL production in all isolates of *P. aeruginosa* by CDST using MEM and molecular characterization of the MBL gene. However, further investigation in a multicentre trial is needed before this is advocated as a gold standard.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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