

DETECTION OF MBL PRODUCTION IN PSEUDOMONAS AERUGINOSA IN KATIHAR DISTRICT, BIHARTarannum Yasmin¹, Krishan Nandan², Aninda Sen³¹Associate Professor, Department of Microbiology, Katihar Medical College, Bihar.²Associate Professor, Department of Microbiology, Katihar Medical College, Bihar.³Professor, Department of Microbiology, Katihar Medical College, Bihar.**ABSTRACT****BACKGROUND**

Pseudomonas aeruginosa is a leading cause of nosocomial infection due to its numerous intrinsic and acquired mechanisms of drug resistance. Although antibiotic resistance in *P. aeruginosa* is caused by multiple mechanisms, one major factor leading to resistance is the production of carbapenemases. Metallo- β -lactamases (MBLs) are carbapenemases which require zinc at the active site and are predominantly produced by *P. aeruginosa*. The selection criteria for confirmation of MBL producers are reduced susceptibility or resistance to carbapenems and/or ceftazidime.

The aim of the study was to determine the efficacy of three antibiotics viz., meropenem, imipenem and ceftazidime as a screening and confirmatory agent for detection of MBL and to assess the diagnostic potential of DDST, DPT and MIC reduction test for detection of MBL.

MATERIALS AND METHODS

This study included 461 isolates of *P. aeruginosa* collected over a period of 2 years. Resistance to carbapenems and ceftazidime was used for screening of isolates. Positively screened isolates were further subjected to different MBL detecting tests- DDST (Double Disc Synergy Test), DPT (Disc Potential Test) and EPM/EPI Microdilution test.

RESULTS

A total of 12304 samples were processed during the study period of which 461 isolates were *Pseudomonas aeruginosa*. Out of 461 *Pseudomonas aeruginosa*, 45.78% were MBL producers. Majority of MBL strains were isolated from Department of Surgery (41.23%). MIC was positive in 211 isolates, being a gold standard test for MBL detection with 100% sensitivity. DPT was positive in 89.57% isolates and DDST was positive in 74.4% isolates. Most MBL strains (93.83%) were sensitive to Polymyxin B.

CONCLUSION

The present study gives us an indication regarding the occurrence of MBL production amongst *Pseudomonas aeruginosa* in Eastern Bihar which is very high at 45.78%. Early detection of MBL producing *Pseudomonas aeruginosa* may help in appropriate antimicrobial therapy and avoid the development and dissemination of these strains. The DPT was found to be a simple, reliable and reproducible test that showed 89.5% conformity. Therefore, we recommend incorporation of DPT in all laboratories as a part of routine.

KEYWORDS

MBL, Pseudomonas Aeruginosa, Carbapenem.

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BACKGROUND

Pseudomonas aeruginosa (*P. aeruginosa*) is a leading cause of nosocomial infection due to its numerous intrinsic and acquired mechanisms of drug resistance.⁽¹⁾ *Pseudomonas* infection is a major cause of concern for treating physicians. Although antibiotic resistance in *Pseudomonas aeruginosa* is caused by multiple mechanisms, one major factor leading to resistance is the production of carbapenemases.

Pseudomonas aeruginosa producing MBLs were first reported from Japan in 1991 and since then have been reported from various parts of the world, including Asia,

Europe, Australia, South America and North America.^(2,3) MBL producing *Pseudomonas aeruginosa* isolates have been responsible for serious infections, treatment failure and several nosocomial outbreaks in different parts of the world resulting in high morbidity and mortality, increased economic burden and an urgent need to establish a strong infection control protocol.

Metallo- β -lactamases (MBLs) are carbapenemases which require zinc at the active site and are predominantly produced by *P. aeruginosa*.⁽⁴⁾ They belong to Ambler's class B and Bush-Jacoby Medeiros Group 3 and hydrolyse virtually all β -lactam agents, including the carbapenems. Further, as the genes coding them are carried on highly mobile elements, their spread in recent years from *P. aeruginosa* to *Enterobacteriaceae*, has led to a situation where a clinical scenario comparable to the global spread of extended spectrum β -lactamases is developing. Till now seven main types of MBLs have been described throughout the world – IMP, VIM, SPM, GIM, SIM, AIM-1⁽⁴⁾ and NDM-1.⁽⁵⁾ Among them, bla_{IMP} and bla_{VIM} are the most common types of MBLs with worldwide distribution.⁽⁶⁾ From India only bla_{VIM}^(7,8) and

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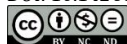
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NDM-1⁽⁹⁾ have been reported in *P. aeruginosa* in the past. VIM (Veronese Imipenemase) enzymes have been grouped into three main clusters designated VIM-1, VIM-2 and VIM-7. To date, VIM-2 is more widely spread among *P. aeruginosa* isolates, whereas VIM-1 is normally confined to *Enterobacteriaceae* isolates.⁽¹⁰⁾ Clinical and Laboratory Standards Institute (CLSI) has not laid down any specific guidelines though there are several screening methods recommended for detection and confirmation of MBL production in *P. aeruginosa*. The selection criteria for confirmation of MBL producers are reduced susceptibility or resistance to carbapenems and/or ceftazidime. Currently, the most widely accepted standardised MBL confirmation method is the MBL Etest (AB Biodisk, Solna, Sweden). However, due to the high cost many clinical microbiology laboratories use alternative methods such as the double-disc synergy test (DDST) and the combined disc (CD)/disc potentiation test (DPT). Although the DDST and the DPT assay are simple to perform and cheaper than the Etest they have shown discordant results, depending on the employed methodology, β -lactam substrates, MBL inhibitors (IMBL), and bacterial genus tested.⁽¹¹⁾ As zinc ion is essential for the action of MBLs, chelating agent like Ethylenediaminetetraacetic acid (EDTA) has been used commonly as *in vitro* inhibitors of MBLs.

This study was conducted to determine the efficacy of three antibiotics viz., meropenem, imipenem and ceftazidime as a screening and confirmatory agent for detection of MBL and also to assess the diagnostic potential of DDST, DPT and MIC reduction test for detection of MBL.

MATERIALS AND METHODS

The prospective study was conducted over a period of 2 years from July 2013 to May 2015 in the Department of Microbiology, Katihar Medical College & Hospital, Katihar, Bihar, India. Patients of both sexes and all age groups were included in this study after obtaining Institutional Ethical Committee clearance.

This study included various clinical samples such as urine, pus, blood and body fluid received for culture. *Pseudomonas* isolated from stool samples were excluded due to their presence as commensal.

All the specimens collected were subjected to direct microscopy, growth on culture media and a series of tests for identification of *Pseudomonas aeruginosa*. After identification, these isolates were subjected to antibiotic susceptibility testing by Kirby-Bauer disk diffusion technique according to Clinical and Laboratory Standards Institute (CLSI) guidelines. All the disks were procured commercially (Hi-Media Laboratories Limited, Mumbai, India). The diameter of the zone of inhibition was measured and interpreted according to the CLSI guidelines.

Screening for MBLs

Isolates of *P. aeruginosa* were considered screen-test positive for MBL if it showed resistance to Imipenem 10 μ g (IPM) and/or Meropenem 10 μ g (MEM) and/or Ceftazidime 30 μ g (CAZ) (HiMedia). Antibiotic sensitivity test was performed by Kirby-Bauer disc diffusion method as per CLSI guidelines.⁽¹²⁾

All positively screened probable MBL producing isolates were subjected to

Double Disc Synergy Test (DDST)

This test was performed as described by Lee et al.⁽¹³⁾ with some modifications. IPM (10 μ g), MEM (10 μ g), CAZ (30 μ g) discs were placed on the Muller Hinton agar (MHA) plates seeded with 0.5 McFarland (10^8 CFU/mL) of test organism with centre to centre distance of 20 mm from a disc containing 5 μ L 0.5M EDTA (930 μ g). Plate should be incubated at 37°C for 18-20 hours. Enhancement of zone of inhibition around IPM and/or MEM and/or CAZ toward the EDTA disc in comparison with the zone of inhibition on the far side of corresponding antibiotic disc was interpreted as positive for MBL production. *Pseudomonas aeruginosa* ATCC 27853 was used as control.

Combined Disc Test/Disc Potentiation Test (DPT)

This test was performed as described by Yong D et al.⁽¹⁴⁾ Two discs each of 10 μ g IPM, 10 μ g MEM and 30 μ g CAZ placed on lawn culture of 0.5 McFarland test organism seeded on MHA. To one disk of IPM, MEM and CAZ 5 μ L of 0.5 M EDTA (930 μ g) was added. Plates were incubated at 37°C for 18-20 hours. After incubation, zone of inhibition was measured using Hi-media scale. Organisms which showed increased zone of inhibition by 7 mm or more (DPT-1: Yong D et al)⁽¹⁴⁾ around any or all of the three discs with EDTA or showed increase by 5-28 mm inhibition around only CAZ-EDTA disc (DPT-2: Hemlatha et al)⁽¹⁵⁾ as compared to IPM, MEM and CAZ discs alone, respectively, considered to be MBL producers. A blank disc of EDTA was tested as control.

EPM/EPI Microdilution Test: EDTA Phenanthroline + MEM/IPM Microdilution Test⁽¹⁶⁾

MIC was determined in 96 well microtitre plates using 50 μ L of Muller Hinton broth, 5 μ L of bacterial inoculums (5×10^4 CFU: Soon after preparation) and 50 μ L of corresponding concentration of the antibiotic per well as per CLSI guidelines.⁽¹⁰⁾ IPM/MEM concentration in the range of 0.25 – 512 μ g/mL was tested. Reduction in MIC of IPM/MEM will be determined by adding 5- μ L mixture of chelators EDTA 0.4 mM and 1, 10-phenanthroline 0.04 mM to the second row, just before inoculation of wells with broth culture. Growth controls without IPM/MEM but with and without chelators mixture were included. Results were recorded by visual inspection of microtitre plates after 18 hours of incubation at 37°C as per CLSI guidelines.⁽¹⁰⁾ The test was considered valid when acceptable growth (More or equal to 2 mm button or definite turbidity) occurred in the positive control well. Absence of turbidity or buttons of less than 2 mm diameter in the test well were thus taken as the MIC of the organism under test. A \geq four-fold IPM and/or MEM MIC reduction in presence of chelators as compared to MIC without them was taken as cut-off value for MBL production.

RESULTS

A total of 12304 samples were received in the laboratory in which 7261 showed no growth. 5043 samples showed growth of different bacterial isolates. 1426/5043 (28.27%) showed growth of GPC in pure culture, 2876/5043 (57.02%) showed growth of GNB in pure culture, 449/5043 (8.90%)

showed mixed growth and 292/5043 (5.79%) showed presence of candida species.

Amongst 2876 GNB isolates, only 461/2876 (16.02%) showed the growth of *Pseudomonas aeruginosa* which were processed for final study. Out of 461 *Pseudomonas aeruginosa*, 211/461 (45.78%) were MBL producers and 250/461 (54.22%) were non-MBL producers.

Out of 211 MBL strains, 118/211 (55.92%) were isolated from male patients and 93/211 (44.08%) were isolated from female patients. Maximum number of MBL producers were seen in age group of 21 – 30 years (36.97%) followed by the age group of 31 - 40 years (20.38%).

Maximum number of cases were MBL producers which were isolated from pus samples 149/211 (70.61%), followed by urine samples 59/211 (27.96%), blood 2/211 (0.94%) and body fluid 1/211 (0.47%).

Majority of MBL strains were isolated from Dept. of Surgery 87/211 (41.23%) followed by Dept. of Orthopaedics 42/211 (19.90%), Dept. of Medicine 39/211 (18.48%), Dept. of Obstetrics & Gynaecology 36/211 (17.06%) and Dept. of Paediatrics 7/211 (3.31%) [Table: 1].

Based on the screening criteria for MBL production, 211 isolates were screened positive. The tests for MBL detection were performed on 211 positively screened isolates. MIC was positive in 211 isolates, being a gold standard test for MBL detection with 100% sensitivity. DPT was positive in 189 (89.57%) isolates and DDST was positive in 157 (74.4%) isolates. In this study, MIC showed the highest sensitivity (100%) while DDST was the least sensitive test (74.4%) [Table: 2].

Among 211 MBL strains, only 198/211 (93.83%) were sensitive to Polymyxin B, 183/211 (86.72%) were sensitive to Colistin. Nitrofurantoin 41/59 (69.49%) was sensitive against urinary isolates only.

Department	MBL		Non-MBL		Total
	IPD	OPD	IPD	OPD	
Medicine	18	21	23	31	93
Surgery	59	28	24	39	150
Orthopaedics	24	18	21	36	99
OBG	17	19	22	33	91
Paediatrics	04	03	07	14	28
Total	122	89	97	153	461

Table 1. Department wise Distribution of MBL and Non-MBL Producers

Name of Test	Positive	Percentage
MIC	211	100
DPT	189	89.57
DDST	157	74.4

Table 2. Comparative Study of Different Methods for MBL Detection (n=211)

DISCUSSION

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.⁽⁸⁾ A case controlled study from Japan showed that patients infected with MBL-producing *P. aeruginosa* were more likely to receive multiple antibiotics and also infection related deaths due to MBL-producing *P. aeruginosa* were

more frequent than the death caused by MBL negative *P. aeruginosa*.⁽¹⁷⁾

In this study, out of 461 *P. aeruginosa* isolates, 211 isolates were screened out as probable MBL producers based on their resistance to carbapenems and CAZ. We found 24.6% resistance among *P. aeruginosa* to IPM and meropenem each while 29.8% resistance to CAZ. A study done at a tertiary care hospital in Puducherry (India) in 2006 reported 10.9% resistance to Carbapenems,⁽¹⁸⁾ while another study in Puducherry in 2008 reported 31.1% resistance to meropenem.⁽¹⁹⁾ A 5-year longitudinal study from Latin America has reported that *P. aeruginosa* resistance to carbapenems has risen to 40%.⁽²⁰⁾ These findings show there is a rising trend in the carbapenem resistance among the *P. aeruginosa*.

Phenotypic tests for MBL have not been nationally or internationally standardised. PCR analysis is the gold standard method for detection of MBL producers, but it is not suitable for daily testing in clinical laboratories due to the cost and inconvenience.⁽²¹⁾

In our study, DPT and DDST also showed good sensitivity for MBL detection as 89.5% and 74.4% respectively. DPT is a rapid and easy performed test, hence we found the DPT to be satisfactory for detecting MBL as it is an easy procedure and is simple to interpret in accordance with other studies.^(14,22,23) Similar to our observation, Behera *et al*⁽¹⁾ and Qu *et al*⁽¹¹⁾ reported that DPT is better than DDST for routine MBL detection.

CONCLUSION

The present study gives us an indication regarding the occurrence of MBL production amongst *Pseudomonas aeruginosa* in Eastern Bihar. Unfortunately, the number isolated by us is alarmingly large (45.78%). It has also been observed that majority of these MBL producers were from indoor patients which indicates that most of these strains are hospital acquired and the presence of these organisms in the hospital environment is a man-made phenomenon due to overuse and misuse of broad spectrum antibiotics. Proper infection control practices and formulation of a hospital antibiotic usage policy is clearly indicated.

The other main issue that needs to be addressed is early detection of MBL producing *Pseudomonas aeruginosa* which will help in appropriate antimicrobial therapy and avoid the development and dissemination of these strains. The DPT was found to be a simple, reliable and reproducible test that showed 89.5% conformity with the MIC reduction test which is time taking, difficult to interpret and needs a high degree of precision. It is recommended that DPT be incorporated in all laboratories as a part of routine antibiotic susceptibility testing procedures especially on strains showing resistance to any one of the carbapenems.

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