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IDENTIFICATION AND PHENOTYPIC CHARACTERISATION OF METALLO – BETA – LACTAMASE PRODUCING PSEUDOMONAS AERUGINOSA ISOLATES AT A TERTIARY CARE CENTRE IN BIHAR

Microbiology

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ABSTRACT

Metallo-beta-lactamase (MBL) producing *Pseudomonas aeruginosa* has emerged as a threat to hospital infection control, due to its multi-drug resistance, especially in intensive care units (ICUs). This study was carried out to detect MBL producing P. aeruginosa isolates, to compare and evaluate different phenotypic methods currently in use and to determine antibiograms. A total of 152 strains were recovered from various clinical samples from patients admitted in inpatient wards and ICUs, and screened for imipenem resistance by Kirby Bauer disk diffusion method. Detection of MBLs was further done by Imipenem/Imipenem- EDTA combined disc diffusion method and E test. Out of 152 isolates, 36 isolates (23%) were imipenem resistant. When further tested for MBL production, six were positive by combined disk test, but, only four showed positive results by E- test. Therefore Routine screening of P. aeruginosa using inexpensive phenotypic tests like imipenem and imipenem + EDTA combined disk test should be an essential component of infection control in hospitals.

KEYWORDS

Metallo-beta-lactamases; infection control; *Pseudomonas aeruginosa*; Multidrug resistance.

INTRODUCTION

Pseudomonas aeruginosa (P.aeruginosa) is one of the most common gram negative pathogen involved in nosocomial infection. It is an important opportunistic pathogen of ICU and hospitalized immunocompromised patients causing a wide range of acute and chronic infections ranging from urinary tract infections to severe sepsis1. The ability of *P. aeruginosa* to survive on minimal nutritional requirements and to tolerate a variety of physical conditions has allowed this organism to persist in both community and hospital settings. Community reservoirs of this organism include swimming pools, whirlpools, hot tubs, contact lens solution, home humidifiers, soil and rhizosphere, and vegetables[2,3]. In the hospital, *P. aeruginosa* can be isolated from a variety of sources, including respiratory therapy equipments, antiseptics, soap, sinks, mops, medicines, and physiotherapy and hydrotherapy pools4.

Despite the wide distribution of *P. aeruginosa* in nature and the potential for community-acquired infections, serious infections with *P. aeruginosa* are predominantly hospital acquired.

The antimicrobial susceptibility pattern found in *Pseudomonas aeruginosa* is unique and worrisome. It has innate resistance to many antimicrobials and disinfectants. Acquired drug resistance in *P. aeruginosa* is very frequent, involving more than one antimicrobial class. Though carbapenems are drug of choice for severe pseudomonas infections (Cornaglia G et al,2007), acquired Metallo – beta-lactamases (MBLs) have recently emerged as one of the most serious resistance mechanism owing to their capacity to hydrolyse almost all beta lactams including carbapenems. MBLs are broad-spectrum enzymes that hydrolyse most of the beta lactam antimicrobial except monobactams and are not inhibited by conventional beta-lactamase inhibitors like clavulanic acid or sulbactam This resistance is further aggravated as because these resistant genes are carried on highly mobile elements along with easy dissemination to different microorganisms. Such strains are not susceptible to therapeutic serine beta lactamase inhibitor such as clavulanic acid and sulphons.

As the MBL producing isolates are mostly seen in tertiary care hospitals in very serious patients, it carries high morbidity and mortality rates. Moreover as the MBLs will hydrolyse virtually all classes of beta lactams and that, we are several years away from development of a safe therapeutic inhibitor, there is every possibility that MBLs will continue to spread. All these could lead to a clinical disaster.

So these isolates in a hospital, cause not only a therapeutic problem but severely challenges infection control program in the said set up. That is why early detection of MBL is very much crucial. The benefits will be obvious for timely implementation of strict infection control practices and treatment with suitable antimicrobials. Unfortunately in most of the hospitals there is no routine set up for MBL detection.

AIMS AND OBJECTIVES

Objectives of the study are to

1. Identify Metallo-beta-lactamase producing *Pseudomonas aeruginosa* from various clinical samples coming to IGIMS, Patna.
3. Determine the prevalence, source and pathogenic distribution of these isolates.
4. Compare double disc diffusion methods and MBL E-test for MBL detection.
5. Determine antimicrobial susceptibility pattern of these isolates.

MATERIALS AND METHODS

A prospective study was carried out at Indira Gandhi Institute of Medical sciences, Patna, Bihar over a period of 18 months from February 2016 to July 2017 after approval of the Institutional Ethics committee.

Consecutive non-duplicate isolates of *P. aeruginosa* from various clinical samples received in the microbiology laboratory for bacterial culture and sensitivity from patients admitted in medical and surgical wards and ICUs were included in this study. Identification of P. aeruginosa was done as per standard laboratory procedures. Antimicrobial sensitivity testing was performed on Mueller-Hinton agar plates by Kirby-Bauer disk diffusion method, according to Clinical Laboratory Standards Institute (CLSI) guidelines. The following antibiotics (Hi-Media, Mumbai, India) were tested by disk diffusion method: piperacillin/ tazobactam (100µg/ 10µg), ceftazidime (30µg), amikacin (30µg), ciprofloxacin (5µg) and imipenem (10µg), gentamicin (10µg), polymyxin-B (300 units), colistin (10µg), Tobramycin(10 µg), cefotaxim (30µg) and aztreonam.

Imipenem disk diffusion method was employed as a screening test to

select suspected MBL- *P. aeruginosa* strains showing resistance to imipenem which were further confirmed by imipenem-EDTA combined disk method and E – test.

Screening for MBL production was done in imipenem resistant isolates by the following methods.

Imipenem (IMP) – EDTA combined disc test

The test organisms were inoculated by lawn culture technique on the plates of Muller-Hinton agar (MHA) as recommended by CLSI. 10 µg Imipenem Disk and 750 µg Imipenem-EDTA Disk (Hi-media SD281) were placed on the plate. The inhibition zones of the imipenem and imipenem-EDTA disks were compared after 16 to 18 hours of incubation at 37°C. In the combined disc diffusion test, if the increase in inhibition zone with the imipenem and EDTA disc was ≥ 7 mm than the imipenem disc alone,it was considered as MBL positive.5

MBL E -Test

The E-test MBL Strip contains a double sided seven-dilution range of IP (Imipenem) (4 to 256 µg/ml) and Imipenem (1 to 64µg/ml) in combination with a fixed concentration of EDTA.It is considered as the most sensitive method for MBL detection. In the study, E-test was done according to manufacturer's instructions.MIC ratio of IP/ IPI (Imipenem+EDTA) of >8 or >3 log dilutions indicated MBL production. 6

Interpretation:

Following interpretative criteria was used for susceptibility categorization.

Report	Formula	Interpretative Criteria
MBL positive strain	IPM / IPM+ EDTA = >8 IPM / IPM+ EDTA = ≥256 / <64 IPM / IPM+ EDTA = ≥256 / <1	When the ratio of the value obtained for Imipenem (IPM) : the value of Imipenem + EDTA (IPM+EDTA) is more than to 8 or If zone is observed on the side coated with Imipenem+EDTA & no zone is observed on the opposite the side coated with Imipenem, interpret the culture as MBL positive.
MBL negative strain	IPM / IPM+ EDTA = ≤8	When the ratio of the value obtained for Imipenem (IPM) : the value of Imipenem + EDTA (IPM+EDTA) is less than or equal to 8
MBL (non-determinative)	IPM / IPM+ EDTA = ≥256 / >64	When no zone of inhibition is obtained on either side. In such cases resistance may be due to mechanisms other

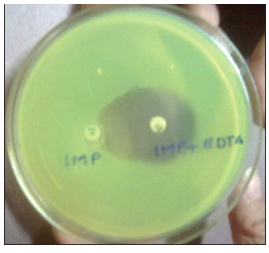


Fig 1.:MBL produced by *Pseudomonas aeruginosa*. detected by combined disc diffusion test

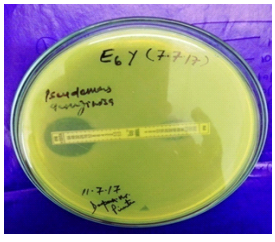


Fig 2- : MBL positive *Pseudomonas aeruginosa* isolate

STATISTICAL ANALYSIS

All statistical analysis were done with Microsoft Excel sheet Window 2007.The results were expressed as percentage for the description of pseudomonas isolatesaccording to species and various clinical samples. Data were statistically analysed during the chi-square test. A value of $p < 0.05$ was considered significant.

RESULTS AND ANALYSIS

A total 152 *Pseudomonas aeruginosa* isolates were obtained in the microbiology lab of IGIMS over a period of 18 months.

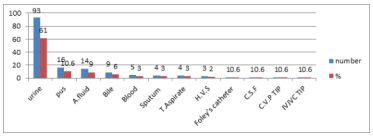
Out of 152 *Pseudomonas aeruginosa* isolates, 23% were imipenem resistant (IMP-R), 29% multi drug resistant (MDR) and only 3% of the isolates were metallo-beta-lactamase (MBL) producers.

Total no. of urine samples was 92. Out of which 68 (44.7%) were males and 24 (15.78%) were females whereas in the culture positive exudate samples out of 60, 38 (63.33%) cases were males and 22 (36.66%) cases were females. Maximum number of urine samples were found in the age group of above 50 yrs. Maximum number exudates sample were found in the age group 21 – 50 yrs i.e in the young, actively working group of patients.

Table 1: Distribution of *Pseudomonas aeruginosa* culture positive cases

Study group	Sex distribution (n=152)			Age in years(n=152)				
	Male	Female	Total	1-10	11-20	21-50	above 50	Total
Urine	68 (44.7%)	24 (15.78%)	92	5 (3.2%)	11 (7.2%)	19 (12.5%)	57 (37.5%)	92
Exudates	38 (25%)	22 (14.47%)	60	3 (1.97%)	6 (3.94%)	29 (19.0%)	22 (14.47%)	60

Chart 1: Sample wise distribution of Pseudomonas aeruginosa



Highest number of samples were urine (62%) followed by exudates(38%). Pus, ascitic fluid and bile made the highest share of exudates. In general, majority of pus samples were obtained from general surgery ,orthopaedics and ENT inpatients.Susceptibility pattern of *Pseudomonas aeruginosa* isolates for different antimicrobials has been shown in Table 2. Maximum sensitivity was found for colistin (95.2%) followed by Polymyxin B, Imipenem, Meropenem, Amikacin, Tobramycin and Ciprofloxacin.

Table 2: Antimicrobial susceptibility of *Pseudomonas aeruginosa* isolates (n=152)

Serial no	Antimicrobial	Sensitive No(%)	Resistant No(%)
1	Imipenem	116(77%)	36(23%)
2	Meropenem	114 (75%)	38(25%)
2	Amikacin	89 (59%)	63 (41%)
3	Tobramycin	89 (59%)	63 (41%)
4	Cefotaxim	53 (35%)	99 (65%)
5	Ceftazidime	60(40%)	92 (60%)
6	Ciprofloxacin	71(47%)	81 (53%)
7	Colistin	145(95.3%)	07 (4.7%)
8	Polymyxin B	144 (94.8%)	08(5.2%)
9.	Aztreonam	96(63%)	56 (37%)

Out of 36 imipenem resistant strains of *Pseudomonas aeruginosa* , 6 showed M B L production by C D D T method, whereas only 4 MBL producers could be detected by MBL E-Test. So combined disc diffusion test was found to be more sensitive.

Table 3: Number of imipenem resistant and MBL producing *Pseudomonas aeruginosa* isolates by CDDT and E-Test (n=152)

Total no. of isolates	Imipenem resistant	Method of MBL detection	
		CDDT	E-Test
152	36	6	4

DISCUSSION

In various studies across the world, varying rates of resistance (4-60%) have been reported for imipenem and meropenem.[7] Of the Indian workers, Gladestone et al reported 42.8% carbapenem resistance among P.aeruginosa isolates.[8] Taneja et al reported that 36.4% of nosocomial urinary tract infections were caused by nonfermenters resistance to imipenem[9] .The prevalence of imipenem resistance , in the present study was 23%. It is comparable to above studies. This is due to indiscriminate and wide spread use of imipenem / meropenem, cephalosporins, piperacillin / tazobactum and fluoroquinolones in the hospital.

In the present study, MBL producing *Pseudomonas aeruginosa* isolates were found to be 3% only. It is comparable with few Indian

studies done by Gibb AP et al 2002, John S et al 2011. On the other hand Ibukun et al [10] Prashant et al., [11] Agarwal et al., [12] Jayakumar et al., 13Navneeth et al., [14] and Variya et al., found it to be 10-25%[15]. One Indian study has reported very high prevalence (80%) Yong et al.

The percentage variation in the resistance mechanism could be due to the study environment . The carbapenem agents are important drugs for treatment of infection; however, indiscriminate use of these drugs may promote their resistance .

Among the β lactam drugs, Ceftazidime (60%), and Cefotaxime (65%) showed the highest resistance in the present study. K.M Mohanasundaram et al., (84.6%) [16], Yapar et al., (84%) and Ibukun et al., (79.4%) [17], reported more resistance against ceftazidime in their study. Our study is in line with the reports of Diwivedi et al., (63%) & Arya et al., (55.4%) [18]. Cause may be Indiscriminate use of 3rd generation cephalosporin as broad spectrum empirical therapy and enzymes mediate the resistance by hydrolysis of β-lactam ring of β-lactam antimicrobials. Other mechanisms of drug resistance to β-lactam group of antimicrobials are loss of outer membrane protein, production of class C AmpC β-lactamase and altered target sites.

Ciprofloxacin showed 53% resistance in our study. Various reports on ciprofloxacin resistance to P.aeruginosa ranged between 0-89% (Algun et al.,) [19]

Multi Drug Resistant *P.aeruginosa* (MDR PA) is defined as isolates resistant to at least 3 classes of drugs in anti-pseudomonal cephalosporins, carbapenem, aminoglycosides and fluoroquinolones. MDR is a growing clinical problem, which is recognized as a threat to public health in causing significant on morbidity and mortality and increased economic burden which stems from the misuse of antimicrobials particularly excessive use. The percentage of MDR PA in India ranges from 11.36% reported by Siti Nur Atiquah Idris et al., [20] to 91.6% reported by S.Panranjothi et al., [21]. In our study, 29% P.aeruginosa were found to be Multi drug resistant (MDR) which is comparable with above studies.

Aztreonam has sensitivity of 64% in this study which is comparable with scully BE .et al study (86 %).

Male preponderance (73.9%) was noted in present study . Similar observations were made by, Anupurba et al., (60%) [22] & Siti Nur et al. (57%) [20]. The preponderance of males can be explained by greater number of cases from surgery & orthopaedic wards which usually have more admissions of male patients in our hospital. Other authors also had similar findings (Tsakris et al).They detected 93.3% patients of MBL-PA were male and considered male gender as an independent high risk association.

This study found maximum number of cases in the age group of above 50 yrs in case of urine samples and that of exudates samples in the age group of 21 – 50 yrs. This is in accordance with other studies reported by Okon K.O et al., (24.6%)[23]and Anupurba S et al., [22](45.88%), the common age group was between 21–40 in these studies too.

In recent years,*Pseudomonas aeruginosa* infections in hospitalized patients has been on increase. This increase has been associated with multiple risk factors (Infection Control Fact Sheet of 2007). Four or more such risk factors were found in patients with MBL-positive isolates. These risk factors may be hospital stay > 8 days and treatment in ICU , catheterization, IV line, previous antimicrobial use . and interventions like mechanical ventilation and endotracheal intubation . All the above were major risk factors for imipenem resistance also. In this study also, we reported maximum of *Pseudomonas aeruginosa* culture positive isolates from admitted patients.

In the study, majority of pus samples were obtained from general surgery ,orthopaedics ,and ENT inpatients Ulcerative lesions were predominant in surgery cases. Similarly all medicine, ICU & chest medicine patients had either primary lung disease or developed respiratory co-morbidity. These findings are in keeping with other studies where P. aeruginosa was found frequently to cause respiratory & suppurative skin infections (Zavascki AP et al,2006).

Since there is no standard guideline for detection of MBL, different studies have reported the use of different methods.MBLproduction has been previously reported from India.Most studies have used IPM-EDTA combined disc, double disc synergy test using IPM-EDTA and modified Hodge test. According to those studies MBL production in *P. aeruginosa* ranged from 7%-65%.

For MBL detection, PCR is a highly accurate and reliable process . But due to its high cost and requirement of technically sound laboratory personnels, its accessibility is often limited to reference laboratories. Therefore in resource limited country like ours, we must have to opt for some nonmolecular techniques, taking advantage of enzyme's zinc dependence by using chelating agent such EDTA. In our study we have found that both CDDT and E-Tests can be carried out to detect MBL producer .

In this study, we have used two different methods of screening for MBL production. But we opine that with the Imipenem and EDTA combined disc test with a cut-off >7 mm, the positive and negative results were more clearly discriminated.

The combined disc method using imipenem + EDTA was found to be more susceptible. This is in accordance with other published studies, which have found the combined disc method to be one of the most sensitive techniques for detectingMBL.[24]

We also found the MBL E test to be susceptible for detection of MBL on P. aeruginosa. In this study, We found that all 6 isolates found to be MBL positive by combined disc were not positive with the E –test (two were negative). So it was found to have slightly less sensitivity as compared to combined disc method. This is in accordance with other published studies, which have found the combined disc method to be one of the most susceptible technique for detecting MBL. (Yan JJ, Tsai SH, Chuang CL. Comparison of the double – disk, Combined – disk, and E Test methods for detecting metallo – β – lactamases in gram-negative bacilli. Diagn. Microbiol. Inf. Dis. 2004; 49 : 5 – 1). In a similar study by B. Behera et al. in All India Institute of Medical Sciences, New Delhi in 2008, they also concluded the same.

The E test MBL strip, based on a combination of a β lactam substrate and a β lactam/ metallo β lactamase inhibitor is specifically designed to detect as many clinically relevant MBL as possible.25 The E test MBL strip (IP-IPE) has the ability to detect metallo β lactamases, both chromosomally and plasmid mediated, in aerobic and anaerobic bacteria.25 This novel method could be used by clinical laboratories to monitor the emergence of metallo β lactamase in a range of clinically significant bacteria and by surveillance network to establish the spread of the enzyme.

Both combined disc and E test were found to be more or less equally susceptible for MBL detection. However, given the cost constraints of E test, a simple screening method like combined imipenem/imipenem + EDTA method can be used. The technique is very easy, economical and can be incorporated into the routine testing of any busy microbiology laboratory.

Polymyxin resistance is uncommon among *P. aeruginosa* and several studies reported MDR strains where polymyxin was uniformly susceptible (Williams JD et al 1999, Nouer SA et al2005). Diverse resistance pattern were described by different workers (Sharma M et al 2010). In present study the polymyxin resistance is 5%. All MBL producing P. aeruginosa isolates (100%) were susceptible to polymyxin B. Our findings of 95% susceptible with both polymyxin B and colistin were very much consistent with many studies reported in literature (Marchiaro et al., 2005; Navaneeth et al., 2002; Varaiya et al., 2008; Agarwal et al.,2006.. In absence of therapeutic MBL inhibitors, polymyxins have shown to be effective in the treatment of MDR P. aeruginosa infections. It has been claimed that polymyxins are not as toxic as previously thought (Markou et al., 2003). However, they should not be used in monotherapy. A combination therapy must be preferred.

CONCLUSION

Emergence of MBL producing P.aeruginosa in hospitals is alarming and reflects excessive use of carbapenems. Intensity of selection pressure for usage of broad spectrum antibiotics is high in ICUs, resulting in eradication of competitive flora and selection of multidrug-resistant strains. Therefore a strict antibiotic policy should be followed

in intensive care areas to prevent further spread of MBLs. Clinicians should prescribe antibiotics judiciously. Timely implementation of strict infection control practices and antibiotic resistance surveillance programs should be carried out from time to time. Detection of MBLs by combined disk diffusion method should be routinely performed in all microbiology laboratories for all imipenem-resistant isolates, which will help to reduce morbidity and mortality in these patients.

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