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Original Research Article

Detection of Metallo-Beta-Lactamases Producing *Pseudomonas aeruginosa* which are Received from the Patients in our Laboratory at Dmch, Laheriasarai, Bihar

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Abstract:

Aim: *Pseudomonas aeruginosa* is a gram negative bacillus that produces metallo-β-lactamases (MBLs) and causes several nosocomial infections. The present study was carried to identify such MBL-producing strains in patients.

Methods: From March 2021 to February 2022, the current study was conducted at the Darbhanga Medical College and Hospital in Laheriasarai, Bihar. The clinical isolates and the MBL prevalence were detected using 3 phenotypic methods namely the Combined disc test, Double disk synergy test, and Modified Hodge test.

Results: Total of 85consecutive isolates of *P. aeruginosa* were isolated from various clinical samples. The present findings revealed that 41 to 50 years aged patients produced the highest percentage (24.71%) of isolates. Compared to a urine sample, a pus sample made up the majority of samples (55.29%). Overall Cefotaxime has maximum resistance of 70.58% followed by Cefazidime 67.05%, Gentamicin 55.29%, Amikacin 38.82%,Tobramycin 41.17%, Piperacillin- Tazobactum 37.65% and Imipenem has 18.81% resistance among isolates. In 45.88% isolates, MBL production was present. MBL producers made up 81.25% of the imipenem-resistant isolates. Among MBL producers Cefotaxime, Ceftazidime and Imipenem showed highest resistance whereas the least resistant was found in Piperacillin, Tazobactum and Polymyxin-B.

Conclusion: The initial basic screening tests would be crucial for identifying new drug strains and would help in creating an antibiotic policy for a specific region.

Keywords: *P. aeruginosa* , Metallo-β-lactamase, Modified Hodge test, Resistance, Combined disc test, Double disk synergy test.

Introduction

A member of the Pseudomonadaceae family, *Pseudomonas aeruginosa* is a gram-negative 0.5-0.8 mm by 1.5-3 mm sized rod bacterium. Recent epidemiological studies, especially isolates with high levels of antibiotic resistance has confirmed that these are associated with several nosocomial infections [1]. In order

to mount an infection, it takes advantage of weaknesses in the host's defense system and act as an opportunistic pathogen. Rarely does the bacteria infect healthy tissues; nonetheless, it can assault any tissue that is immunocompromised. *P. aeruginosa* primarily infects the urinary tract, respiratory system, dermis, soft tissue, bacteremia, bone and joint, gastrointestinal tract, and blood in individuals with severe burns, tuberculosis, cancer, and AIDS. Importantly, *P. aeruginosa* has a 50% death rate and poses a serious risk to hospitalised cancer, cystic fibrosis, and burn patients [2].

This ubiquitous flagellated unicellular bacteria has an oxygen-based respiratory system, but it can still grow in the presence of NO3 even in absence of oxygen [3].It utilizes ammonium sulphate (nitrogen source) and acetate (carbon source) as its primary nutrient. While 37°C is the optimal temperature for growth, *P. aeruginosa* can tolerate temperatures as high as 42°C [4]. *P. aeruginosa* cannot be killed by potent antiseptics, concentrated salts, and dyes, or a number of commonly used antibiotics. Both wild-type and clinical strains can exist either as planktonic or biofilm forms [5].

P. aeruginosa isolates display colonies in three different configurations. Clinical isolates are probably smooth colony types, occasionally with a big, smooth, flat-edged, and elevated look like a fried egg. Small, unorganized colonies are typical characteristics of natural wild-type isolates from soil or water. Alginate slime, which resembles mucoid secretions from the urinary and respiratory tracts, may be present. It produces two soluble pigments: pyoverdin, a bright pigment, and pyocyanin, a blue pigment. Pyocyanin, or blue pus, is a characteristic of supportive infections caused by *P. aeruginosa*. Pyochelin, a pyocyanin derivative, is a siderophore that can collect iron from the host or low-iron environments to support the pathogen's growth. Pyocyanin can interfere with human nasal cilia and the respiratory epithelium's normal function, causing pro-inflammatory reactions. [6].

According to the Centers for Disease Control and Prevention (CDC), it is the fourth most frequently isolated nosocomial pathogen, accounting for 10% of all hospital-acquired infections and has an overall infection frequency in US hospitals of 0.4%. After being originally discovered in Japan in 1991, *Pseudomonas aeruginosa* which produces Metallo-β -lactamases (MBLs) has now been widely discovered across Asia, Europe, Australia, South America, and North America. Ambler class B metallo-carbamases are capable of hydrolyzing a wide range of Metallo-β-lactam drugs, including penicillins, cephalosporins, and carbapenems.

Metal chelators like EDTA and substances containing thiols block these enzymes, which need zinc for catalytic activity. The MBL-producing genes are commonly carried on transferable plasmids as integron structures, though they can also be found on chromosomes. Therefore, *P. aeruginosa* isolates producing MBLs are frequently resistant to diverse classes of antimicrobial drugs because of the integron-associated gene cassettes, which can be passed on to different types of bacteria.

According to their molecular architectures, acquired MBLs can be categorized into four groups: IMP, VIM, GIM, and SPM kinds. Three subclasses of class Beta-lactamases (B1 to B3) were discovered based on their sequences, and a uniform numbering system was suggested [7, 8]. *P. aeruginosa* isolates that produce metalloprotease have been linked to a number of nosocomial outbreaks in tertiary care facilities around the globe, underscoring the importance of using effective infection control procedures. These isolates have also been linked to the failure of carbapenem therapy and have caused serious illnesses such as septicemia and pneumonia [9].

Materials and Method

Study setting: The present study was

conducted in the department of microbiology at Darbhanga Medical College and Hospital (DMCH), laheriasarai, Bihar from March 2021 to February 2022 in order to detect metallo beta-lactamases producing *Pseudomonas aeruginosa*.

The study was initiated after obtaining the ethical clearance from the institute and receiving a written consent from the patients. Only in-patients were included in the present investigations. The clinical specimens were isolated from pus and urine samples of the patients. In addition to their antibiogram tests, the Imipenem resistance-displaying isolated strains were exclusively examined to see out how often MBL is produced.

Collection and processing of various samples: All samples were taken under aseptic conditions using conventional techniques, and they were all handled in accordance with recognized best practices. Straight smear test: Gram-stained direct smears were used to check for inflammatory cells and the kind of microbial flora. The gram-stained smear reveals pus cells and gram-negative bacteria. For blood culture, Brain Heart Infusion broth was employed. The bottle was thoroughly checked for turbidity. Subcultures were prepared on blood agar and MacConkey's agar at regular intervals, and any growth was further processed for identification biochemically and via physical characteristics. For identifying the *P. aerogenosa* the culture was grown on nutrient media such as nutrient agar, MacConkey's agar, and blood agar. Their physical characteristics including their texture, smell, type of colonies, visualization after staining were monitored.
Methods used for detection of MBL production:

Modified Hodge test (MHT): On a Mueller Hinton agar plate, a lawn culture of 1:10 dilution of 0.5 McFarland's standard E. coli ATCC-25922 broth was cultured for MHT. Imipenem-resistant *P. aeruginosa* was streaked in 4 distinct directions from the edge of the Imipenem disc to the plate's edge after 10 g of Imipenem and 50 mM zinc sulphate solution (10 l) were added to the Imipenem disc. The plates were examined for a distinctive cloverleaf-shaped depression after an overnight incubation, and those with such zone of inhibition were considered to have a positive MHT result.

Imipenem (IMP)-EDTA combined disc test (CDST): IMP-EDTA combined disc test was performed as per Yong et al.'s instructions. The test organisms were inoculated onto Mueller Hinton agar plates. For this, 18.61 g Mueller Hinton agar was dissolved in 100 ml distilled water. pH 8.0 was achieved by adding 0.1 M NaOH then a 0.5 M EDTA solution was added. The mixture was autoclaved to make it sterile.

To achieve the desired concentration (750 g), the appropriate volumes of 10 L of EDTA solution were added to one of two 10 g imipenem discs (Becton Dickinson) placed on the plate. After 16 to 18 hours in the air at 35°C, the inhibition zones of the imipenem and imipenem-EDTA discs were compared. For CDST positive plates, Imipenem and EDTA discs would have more than 7 mm inhibition zone as compared to the Imipenem disc alone.

IMP-EDTA double disk synergy test (DDST): According to Lee et al.'s instructions, the IMP-EDTA double disc synergy test was carried out. As per the CLSI's advice, test organisms were inoculated onto Mueller Hinton agar plates. A blank disc holding 0.5 M EDTA was positioned at 20 mm distance from the centre of another disc containing imipenem (10 g). The zone of inhibition is enhanced by >5 mm in the space between imipenem and the EDTA disc was observed for positive results.

Antibiotic susceptibility testing: In accordance with CLSI recommendations, the isolates were tested for antibiotic susceptibility using the Kirby Bauer disc diffusion method. The antibiotics

Cefotaxime, Ceftazidime, Gentamicin, Amikacin, Tobramycin, Ciprofloxacin, Imipenem, Piperacillin- tazobactum and Polymyxin-B were purchased commercially from Hi-Media Laboratories Ltd., Mumbai, India and were tested for susceptibility in the current study. The zone's diameter was measured and analyzed in accordance with CLSI standards (Table 1).

Table 1: Antibiotic susceptibility testing as per CLSI

Antibiotic discs	Concentration (µg)	Sensitive zone (mm)	Intermediate zone (mm)	Resistant zone (mm)
Cefotaxime	30	>23	14-23	<14
Ceftazidime	30	>18	14-18	<14
Gentamicin	10	>15	12-15	<12
Amikacin	30	>17	14-17	<14
Tobramycin	30	>15	12-15	<12
Ciprofloxacin	5	>21	15-21	<15
Imipenem	10	>16	13-16	<13
Piperacillin-tazobactum	100/10	>18	-	17
Polymyxin-B	50	>12	-	11

Results

In the present study, clinical samples from pus and urine were collected from 85 patients from March 2021 to February 2022 at DMCH, Laheriasarai, Bihar. The anti-biogram and MBL production of these isolates were investigated.

Distribution of patients as per their age, sex and study sample specimens obtained from patients: The study group's participants range in age from 11 to 90 years old. The majority of the isolates (24.71%) belonged to the 41–50 age range followed by 61-70 age group (21.18%) and 51-60 years (20.00%). Total 16.47% of isolates were obtained from 71-80 years patients. Least number of isolates (1.18%) were obtained from 11-20 years (Figure 1 a).

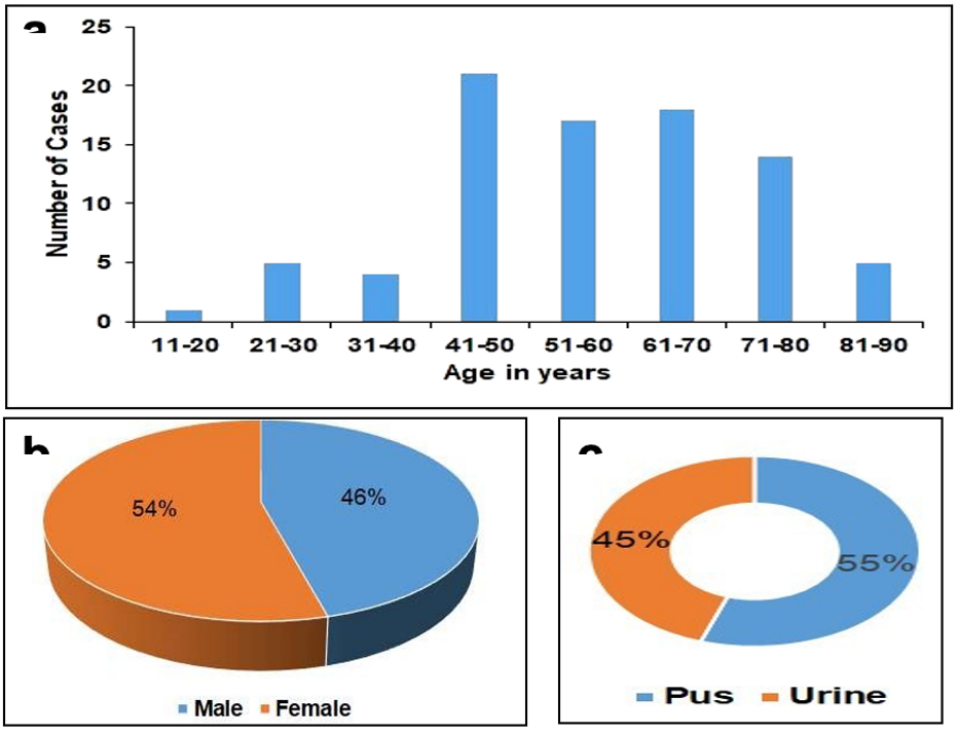

Figure 1: Distribution of patients according to their (a) Age, (b) Sex, (c) Samples specimens

Total 39 male (45.88%) and 46 female (54.12%) were included in study for isolation of bacterium (Figure 1 b). Figure 1 c reveals that out of 85 cases, isolates were obtained from 47 pus samples (55.29%) and 38 urine samples (44.71%).

Identifiation of *Pseudomonas aeruginosa* colonies: The colonies cultured on plates were looked for their distinctive characteristics on different media (Figure 2). The culture on nutrient agar showed water soluble greenish colored colonies due to the production of pyoverdine pigment (Figure 2 a, Figure 3 b). On MacConkey's agar, irregular, flat colonies with bluish-green non-lactose fermenting colonies were observed (Figure 2 b). On the blood agar media dark and deep-colored, flat irregular colonies of bacteria with β-hemolysis was observed (Figure 2 c). Colonies had a particular fruity odor.

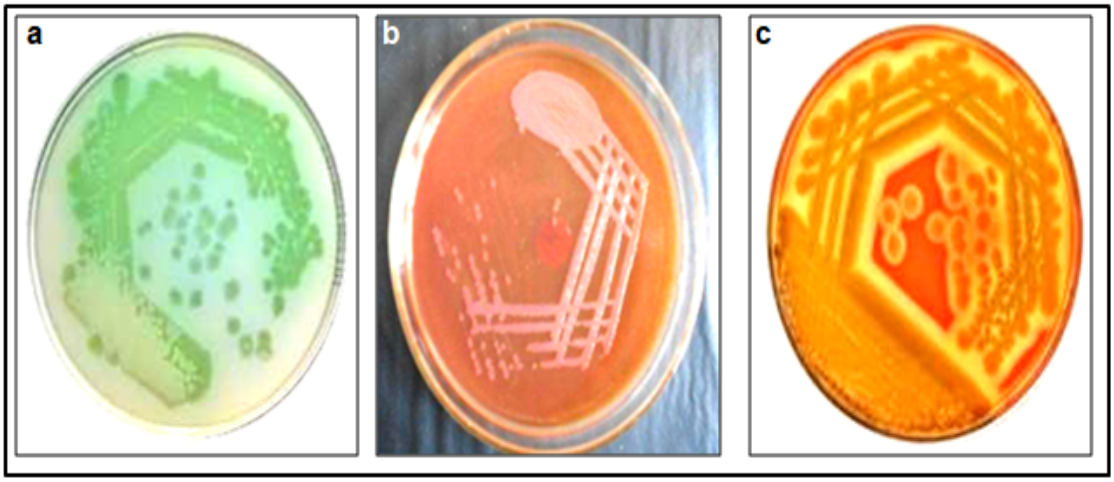

Figure 2: *Pseudomonas aeruginosa* culture on different media (a) Nutrient agar plate showing production of water soluble green colonies due to pyoverdin pigment, (b) Non-lactose fermenting colonies on MacConkey's agar, and (c) Colonies on blood agar showing β-hemolysis

Gram staining and other biochemical tests were performed for further identification (Figure 3, Table 2).

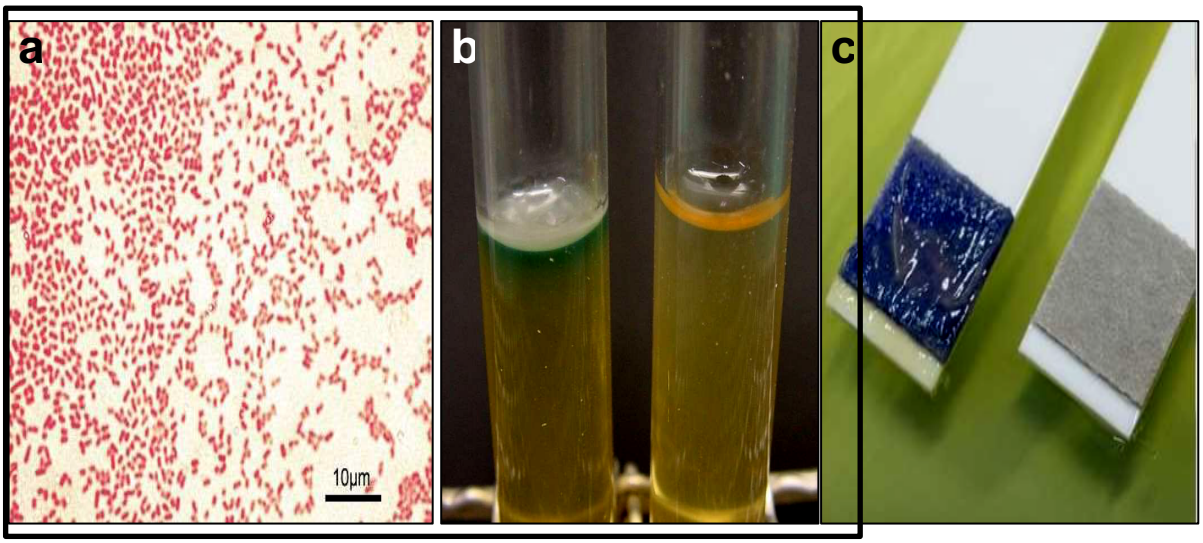

Figure 3. (a) Gram-negative stained *Pseudomonas aeruginosa* colonies, (b) Left tube showing water soluble green pigment, and (c) Oxidase test of *P. aeruginosa*

Table 2: Identification of *P. aeruginosa* colonies through biochemical tests

Test	Result
Oxidative –fermentation	Oxidative
Catalase	+
H_2S production	-
TSI	Alkaline slant/nochange
Oxidase	+
Nitrate reduction	+
Indole	-
Citrate	+
Urease	+
Mannitol fermentation	-
Sucrose fermentation	-
Arginine hydrolase	+

'+' shows positive results and '-' shows negative results in the table; H2S: Hydrogen sulphide, TSI: Triple sugar iron agar

MBL detection using MHT, CDST and DDST methods: All the clinical isolates were tested for MBL-production using MHT, CDST and DDST methods (Figure 4). Figure 4 a shows typical cloverleaf inhibition zone as observed in positive MHT method. Colonies formed larger zone of inhibition in Imipenem+EDTA discs than the Imipenem disc alone in CDST method (Figure 4 b). The zone of inhibition >5 mm was observed in the space between imipenem and the EDTA disc confirming positive results for DDST (Figure 4 c).

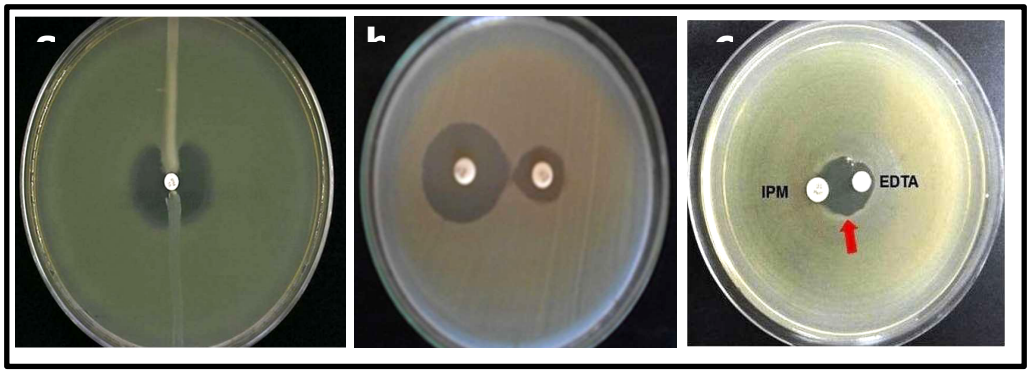

Figure 4: Plate showing positive result for (a) Modified Hogde test, (b) Combined Disk Synergy Test, and (c) Double Disk Synergy Test

Out of 85, total 38 *P. aeruginosa* isolates (45.88%) were detected as MBL producers in the present study. Total 30% carbapenem hydrolysis positivity was observed in case of MHT showing positive result whereas MBL prevalence by the CDST accounted for 80% of cases and DDST for 45% of cases. All tests were negative for *P. aeruginosa* ATCC strain-27853 (Table 3).

Table 3: Detection of Carbapenemases by Modified Hodge test

Tests	Control (ATCC-27853 *P. aeruginosa*)	Number of MBL positive	Percentage positivity
Modified Hodgetest	Negative	6 out of 20	30%
Imp-EDTACDST	Negative	16 out of 20	80%
DDST	Negative	9 out of 20	45%

Detection of Imipenem resistance among various clinical specimens: Out of 47 pus

samples, 9 isolates (56.25%) were resistant to imipenem. In contrast, isolates from 38 urine samples, only 7 (43.75%) were found to be imipenem resistant. In total, 16 Imipenem resistant isolates were obtained in the present study. Out of these isolates, 13 *P. aeruginosa* isolates (81.25%) were positive for MBL-production and 3 (18.75%) were non-MBL producers.

Table 4 reveals the total imipenem resistant *P. aeruginosa* isolates and number MBL-producers among them.

Table 4: Production of MBL by *P. aeruginosa* that is imipenem resistant

Clinical specimens	No. of isolates	MBL production	Percentage%
Pus	9	7	43.75
Urine	7	6	37.50
Total	16	15	100

Antibiotic susceptibility testing

Kirby Bauer disc diffusion method was employed for testing susceptibility against various known antibiotics. Their zone of inhibition was measured and analyzed as per CLSI standards (Table 1) and their resistance was predicted. Figure 5 a depicts the resistance pattern of P.

aeruginosa clinical isolates in the present study. The resistance was observed in 60 (70.58%) isolates against Cefotaxime, 57 (67.05%) isolates against Ceftazidime, 47 (55.29%) isolates against Gentamicin, 33 (38.82%) isolates against Cefotaxime60 (70.58%) isolates against Cefotaxime60 (70.58%) isolates against Amikacin, 35 (41.17%) isolates against Tobramycin, 44 (51.76%) isolates against Ciprofloxacin, 16 (18.82%) isolates against Imipenem, 32 (37.65%) isolates against Piperacillin+Tazobactum (70.58%) and only 1 (1.17%) isolates resistant against Polymyxin-B.

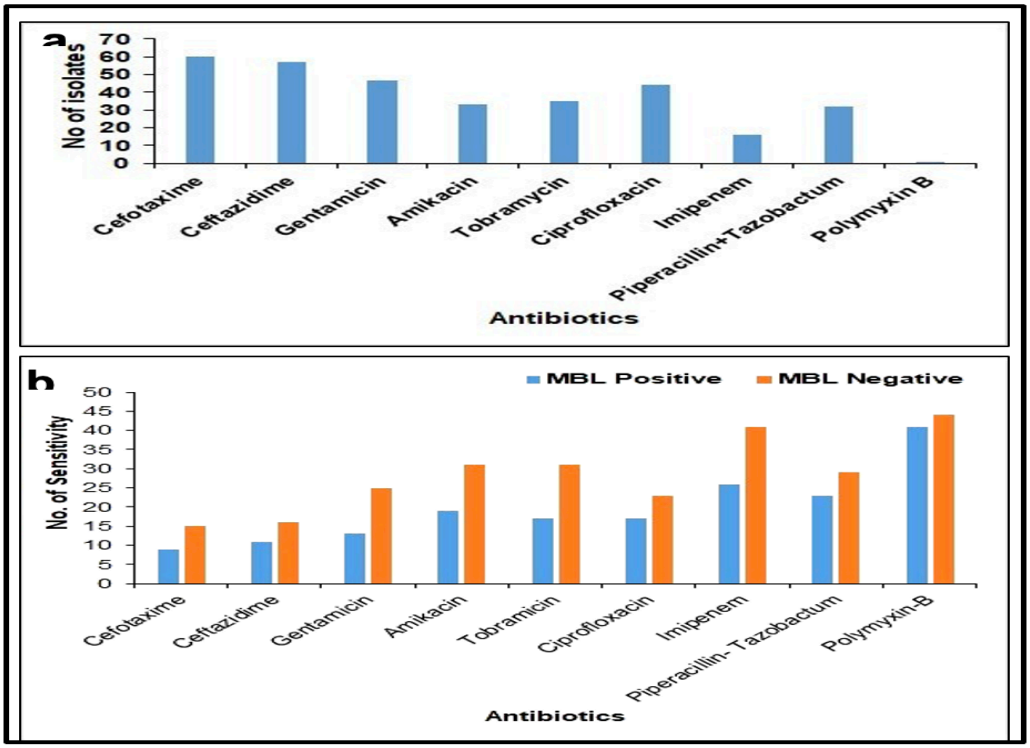

Figure 5: (a) Resistance pattern and (b) Sensitivity pattern of MBL positive as well as MBL negative producing *P. aeruginosa* isolated in the present study

MBL producers were highest sensitive to Polymixin-B. But isolates were least sensitive or resistance to Cefotaxime however in MBL negative isolate showed highest sensitive to Cefotaxime and least sensitive to Polymixin-B (Figure 5 b, Table 5).

Table 5: MBL positive and MBL negative generating isolates' sensitivity patterns

Antibiotic	MBL positive (n= 41)		MBL negative (n= 44)	
	No	Percentage %	No	Percentage %
Cefotaxime	12	23.68	15	34.09
Ceftazidime	11	28.94	16	36.36
Gentamicin	13	34.21	25	56.81
Amikacin	19	50.00	31	70.45
Tobramicin	17	44.73	31	70.45
Ciprofloxacin	17	44.73	23	52.27
Imipenem	26	68.42	41	93.18
Piperacillin-Tazobactum	23	60.52	29	65.90
Polymyxin-B	41	100.00	44	100.00

The resistance pattern was observed against aminoglycosides and ciprofloxacin among MBL producers and it was observed that among all 38 MBL producers in the current investigation, resistance to gentamicin was found to be 65.78% and resistance to ciprofloxacin to be 55.26%. Tobramycin resistance was found to be 55.26% and amikacin resistance to be 50% (Figure 6).

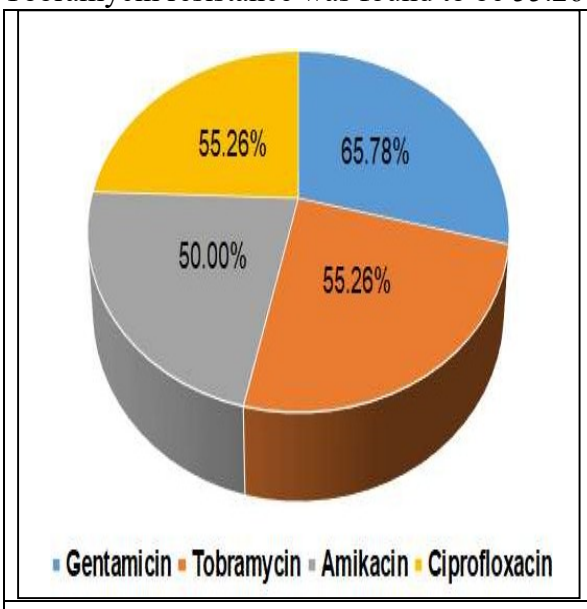

Figure 6: Percentage of MBL producers for Aminoglycosides and Ciprofloxacin

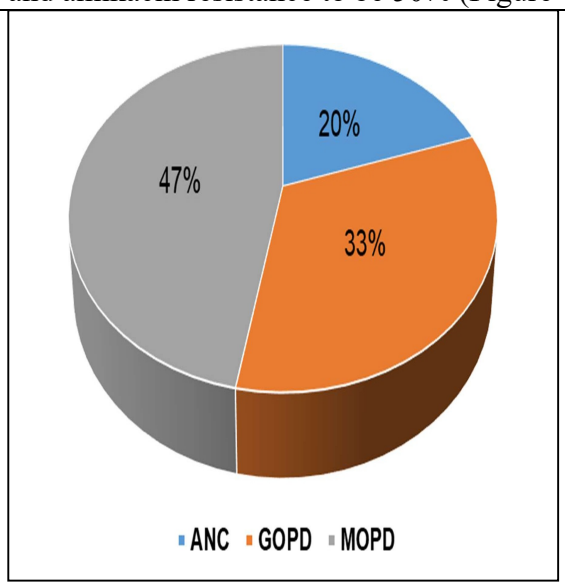

Figure 7: Percentage of cases in various wards

Distribution of cases in various wards

It was observed that the majority of cases (40, 47.05%), were from the Medical out-patient department (MOPD ward), followed by 28 (32.94%) and 17 (20%) from the General out-patient department (GOPD ward) ward. The remaining 17 (20%) samples came from the Antenatal care unit (ANC ward) (Figure 7).

Discussion

The goal of the current study was to determine the frequency of MBL-producing *P. aeruginosa* isolated from pus and urine as well as their antibiogram at DMCH, Laheriasarai, Bihar.

In the present study, total 85 patients aged between 11 and 90 years were included in study. Of these cases, maximum isolates was obtained from patients aged 41-50 years (24.71%). The present finding was in line with Maharjan et. al; 2022 results who reported 27.94% *P. aeruginosa* isolates isolated from 41 to 60 years patients [15]. Javiya et. al; 2008 studied patients of 21 to 60 years and isolated 61.6% isolates [16]. Srinivas et. al; 2012 also isolated 66.67% *P. aeruginosa* isolates from 21 to 60 years old patients [17]. In the present study, the male:

female ratio was 1.18:1. Ninama et. al; 2012 [18], Nasreen et. al; 2015 [19], Jawad et. al; 2016 [20], and Zeb et. al; 2017 [21] also reported that 1.56: 1, 1.61:1, 1.25: 1 and 2:1 male:female ratio in their studies respectively.

In the current investigation, isolates were obtained 55.29% from pus samples and 44.71% from urine samples. However, Arora et. al; 2011 [22] isolated 44% and 36% from pus and urine samples respectively.

The present analysis observed that 70.58% were resistant to Cefotaxime and 67.05% were resistant to Ceftazidime. Similarly Behera et. al; 2008 [23] also reported 78% and 67% resistance against Cefotaxime and Ceftazidime respectively. Dwiwedi et. al; 2009 [12] revealed 90% and 85% resistance against Cefotaxime and Ceftazidime respectively. Bashir et. al; 2011 [14] mentioned 63% and 35% resistance against Cefotaxime and Ceftazidime respectively in the study.

On analyzing the resistance against Ciprofloxacin and Amikacin, present study showed 51.76% and 38.82% resistance respectively. Almost 62.5% and 60% resistance against Ciprofloxacin was reported by Mohanasoundaram et. al; 2011 [24] and Angadi et. al; 2012 [25] respectively. about 42.4% resistance against Amikacin was observed in the study by El-Far et. al; 2021 [26].

Impenem resistance was observed in 18.82% cases in the present stusy. In congruence to the present analysis, Bashir et. al; 2011 [14], Angadi et. al; 2012 [25] and Peshattiwar et. al; 2011 [27] also reported 13.42%, 21.6% and 20.62%impenem resistance respectively.

MBL prevalence in the present study was observed to be 45.88%. Manoharan et. al; 2010 [13], Behera et. al; 2008 [23] and Dogonchi et. al; 2018 [28] observed 42.6%, 39.56% and 38.4% prevalence of MBL-producers in their study respectively.

In present investigation, 81.25% were MBL producing Imipenem resistant strain and 30% were Imipenem-resistant isolates generating carbapenemase. The MBL producing Imipenem resistant of present study which is not closely reported to previous work however the study is close in agreement with study of Behera et. al; 2008 [23] (64.28%) and Gupta et. al; 2012 [29] (69.85%). The Imipenem-resistant isolates generating carbapenemase was reported to be 28.1% by Ahmed et. al; 2020 [30].

The present study showed 80% and 45% sensitivity for CDST and DDST respectively. The sensitivity of the present study agreed with the previously reported study by Behera et. al.,2008 [23] who reported 88.8% sensitivity for CDST and 57.14% for DDST. Das et al., 2022 [31] also showed 80% and 82.6% sensitivity for CDST and DDST respectively.

Total 100% sensitivity towards polymyxin was observed in the present study. Panchal et. al; 2017 [32] and Choudhary et. al; 2019 [33] also reported 92% and 86% polymyxin sensitivity in their study respectively.

In the current experiment, all imipenem-resistant isolates were assessed for MBL production using the combined disc synergy test (CDST) and double disc synergy test (DDST). Cases from MOPD ward in present study experienced highest isolates of 47.05%. Angadi et. al; 2012 [25] reported 52.8% cases from the ward.

Conclusion

The present study envisaged that the early detection of MBL-producing *P. aeruginosa* isolates would aid in both the reduction of mortality rates for patients infected it and in the prevention of the spread of such strains inside hospitals. The more accurate way for identifying such strains is CDST-IPM because molecular approaches are not practicable. In typical microbiology laboratories, the CDST-IPM can be used as a practical screening method for detecting MBL synthesis in gram-negative bacilli. The early detection of MBL-producing isolates would be vital for this method. Basic screening tests conducted on a regular basis will help identify new drug strains and help create an antibiotic policy for a specific region.

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