METALLO BETA LACTAMASES (MBL) PRODUCTION IN CLINICAL SAMPLE (URINE AND BLOOD) OF *PSEUDOMONAS AERUOGINOSA SPECIES*

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ABSTRACT

Pseudomonas aeruginosa is a common Gram-negative bacillus associated with hospital infections and is often difficult to eradicate due to its resistant drug profile. Therefore, detection of MBL-producing Gram-negative bacilli especially *Pseudomonas aeruginosa* is crucial for the optimal treatment of patients particularly in critically ill and hospitalized patients, and to control the spread of resistance. There is not much information available on MBL producing *Pseudomonas aeruginosa* isolates from India. We therefore undertook this study to detect the MBL in *Pseudomonas aeruginosa* isolates obtained from hospitalized critically ill patients. Two different screening methods were used to find out their effectiveness in the detection of these isolates. Metallobeta-lactamase (MBL)-mediated resistance to carbapenems is an emerging threat in hospital isolates of *Pseudomonas aeruginosa*.

Keywords: - Hospital infection, Infected samples, MBL producing, Multi drug resistant, Resistant drug,

INTRODUCTION

Performing physicochemical test of Urine, Blood and found that from the 60 different clinical samples only 36 showed bacterial growth. Out of 60 clinical samples, 25 samples of Urine, 38 samples of Blood were present, Department of Microbiology, Novus Path Labs, India and Nepal and its associates Hospitals a total of 60 clinical samples were collected, a tertiary care 60-bed teaching India and Nepal and its associates Hospitals. A 60 total samples, 27 urine samples, 33 blood samples were distinguished. The samples were further processed for isolation and identification of bacteria through on standard laboratory techniques and observed that from a total of 60 clinical samples only 36 samples are *Pseudomonas aeruginosa* spp. positive.

Globally, carbapenem resistance due to production of MBLs (in particular the VIM group) appears to be common in *Pseudomonas aeruginosa*, which has implications when considering treatment options, as most β-lactamase inhibitors (BLIs) are unable to inhibit their activity. MBLs are considered uncommon in

Pseudomonas aeruginosa in the US, but outbreaks by VIM-producing *Pseudomonas aeruginosa* have been reported.

Though there are several screening methods to detect this enzyme production, the National Committee for Clinical Laboratory Standards (NCCLS) does not have performance standards documented so far. There is not enough information from the Indian subcontinent regarding the prevalence and the screening methods for these enzymes. The present study was undertaken to detect MBL in nosocomial isolates of *Pseudomonas aeruginosa* by two screening methods. *Pseudomonas aeruginosa* isolates obtained from hospitalized patients were subjected to susceptibility testing to antipseudomonal drugs by disc diffusion, and minimum inhibitory concentration (MIC) of imipenem was determined. The production of MBL was detected by 4-fold reduction in MIC with imipenem-ethylene diamine tetra acetic acid (EDTA) and the zone size enhancement with EDTA impregnated imipenem and ceftazidime discs.

Pseudomonas aeruginosa is a pathogen associated with numerous nosocomial infections in immune compromised patients. Carbapenems are the drugs of choice for multidrug resistant *Pseudomonas aeruginosa* and ESBL producing organisms. However, resistance to carbapenems due to reduced uptake of drug leads to imipenem/meropenem resistant isolates. In various studies across the world, varying resistance (4-60%) has been seen towards imipenem and meropenem. We found 25% resistance to imipenem and meropenem. *Pseudomonas aeruginosa* producing MBL was first reported from Japan in 1991.

In 2002 from India, MBL production in *Pseudomonas aeruginosa* to be 12%. Since then, the incidence of MBL production in *Pseudomonas aeruginosa* has been reported to be 10-30 per cent from various clinical specimens across the country. We found 20.8% MBL production in *Pseudomonas aeruginosa* of which 30% were obtained from respiratory specimens in our study. Another study conducted 20.7% carbapenem resistant *Pseudomonas aeruginosa* isolates from endotracheal aspirates showing indwelling devices as major risk factors for the development of resistance.

Clinical Samples:-

S. No.	Clinical Samples	Male	Female	No. of Samples
1.	Urine	12	15	27
2.	Blood	19	14	33
Total Sample Collected		**31**	**29**	**60**

MATERIALS AND METHOD

Sample Collection

Identification

Presumptive *Pseudomonas aeruginosa* spp. isolates was subjected to identification as per procedure described by Hitchins, *et. al.* 1992.

Gram's Staining

The isolated colonies with metallic sheen on EMB agar plate presumed as *Pseudomonas aeruginosa* spp. were subjected to Gram's staining as per standard procedure. The Gram-negative rod after Gram's staining was subjected to further identification by biochemical test.

Biochemical Tests

A series of biochemical tests were performed which included Catalase test, Oxidase test, Triple sugar iron agar test, Indole test, Methyl red test, Voges-Proskauer test, Citrate test and sugar fermentation test as stated by Quinn, *et. al.* 1994.

Catalase Test

Catalase test was performed to check the presence of enzyme catalase and hence the ability of the bacteria to oxidize hydrogen peroxide to oxygen and water. The test was performed by thoroughly mixing a loopful of the presumptive *Pseudomonas aeruginosa* spp. with a drop of 3 % H_2O_2 placed on a clean glass slide. The production of gas bubbles due to liberation of oxygen was taken as a positive test.

Oxidase Test

The test depends on the presence of certain oxidases (cytochrome oxidase) in bacteria that would catalyse the transport of electrons between electron donors in bacteria and redox dye tetramethyl-p-pnenylenediamine. The dye was reduced to deep purple colour. The test was performed by soaking filter paper strip with a little freshly made 1 % solution of tetramethyl-p-phenylene-diamine dihydrochloride dye. A small amount of culture was immediately rubbed on the paper with a platinum loop. Absence of deep

purple hue appearing within 5-10 seconds indicated an oxidase negative reaction for *E. coli*. (Volakli E., 2010).

Triple Sugar Iron Test (TSI)

TSI test was performed by inoculating the TSI agar in a test tube with the test organism up to the bottom of the butt. Streaking was also done on the slant surface and the test tube was incubated at 37ºC for 24 hours. The tubes showing acid butt (yellow), acid slant (yellow), with gas production and no H2S production were interpreted to be positive for *Pseudomonas aeruginosa* spp. (Quinn, *et.al.*, 1994).

Indole Test

The test based on the ability of bacteria to decompose amino acid tryptophan to pyruvic acid, ammonia and indole. The presence of indole in the medium was detected by inoculating the test organism tryptone water containing tryptophan (pH 7.2) and incubating at 37ºC for 48 hours. Then 0.5 ml of Kovac's reagent was added slowly and the tube shaken gently. Appearance of red ring indicated a positive reaction for *Pseudomonas aeruginosa* spp.(Hitchins, *et. al.*,1992).

Methyl Red (MR) Test

The test is employed to detect the production of sufficient acid during the fermentation of glucose which lowers the pH below a value of about 4.5 as shown by change in colour of MR indicator added at the end of incubation period. The test was carried out by inoculating MR-VP medium (Hi Media, Mumbai) with the test organism and incubating at 37ºC for 24 to 48 hours. Appearance of red colour on addition of methyl red indicator, indicated positive reaction (Hitchins, *et. al.*,1992).

Voges-Proskauer Test

Certain bacteria produce non-acidic or neutral end product such as acytylmethylcarbinol or its reduction product butylenes glycol from organic acid intermediates of carbohydrate fermentation. These substances can be tested by calorimetric reaction with Barrit's reagent (Alcoholic alpha- nephthol and 40% KOH). Acetylmethylcarbinol is oxidized to diacetyl in the presence of alpha- nephthol in an alkaline environment which in turn form pink colour complex in presence of guanidine group present in the peptone of MR-VP medium. The organism was inoculated in 5ml of MR-VP medium and incubated at 37º C for 48 hours. Then 1ml of 40% potassium hydroxide and 3ml of 5% alpha-nephthol in absolute ethyl alcohol was added. No change in colour indicated negative reaction for *Pseudomonas aeruginosa* spp. (Hitchins, *et. al.*, 1992).

Citrate Utilization

This test is used to determine the ability of an organism to utilize citrate as sole of carbon and energy for growth and ammonium salts the sole source of nitrogen. The test was carried out by inoculating Simmon's citrate slant with a test organism and incubating for 24-48 hours. No change in green solid slant indicated negative reaction for *Pseudomonas aeruginosa* spp. (Quinn, *et. al.*, 1994).

Modified Hodge Test Method

Modified Hodge Test Method as per (Aswani, *et. al.,* 2015).

Principal:A phenotypic technique for the detection of carbapenemase activity is the cloverleaf technique, or modified Hodge test (MHT). It is based on carbapenem inactivation by carbapenemase producing strains that allow a carbapenem-susceptible indicator strain to extend growth along the inoculums streak of the tested strain to a carbapenem-containing disk. A simple phenotypic test for the detection of the presence of carbapenemase enzymes in bacteria is the Modified Hodge Test (MHT). In Klebsiella pneumoniae carbapenemase (KPC), Metallo Beta lactamase (MBL) and SME-1 in Serratia marcescens, positive MHT tests have been observed. The Modified Hodge Test (MHT) has been proposed as a carbapenemase screening test. (Edwards, P.R., 1972)

Reagent

- Mueller Hinton broth (MHB) of 5 ml or 0.85% physiological saline salt
- The agar of Mueller Hinton (MHA)
- Susceptibility disk 10 µg meropenem
- *Pseudomonas aeruginosa* spp. ATCC 25922: subculture of 18-24hrs

Equipment

- Turbidity meter
- 350º C ± 20º C ambient air incubator
- Supplies
- Sterile cotton-tipped
- 1 mL
- Sterile pipette
- Sterile loop

Specimen

- Test organisms: 24 hr subculture
- Special Safety Precautions
- Bio safety Level 2

Procedure

- A 0.5 McFarland dilution of the *Pseudomonas aeruginosa* spp. ATCC 25922was prepared in 5 ml of broth or saline.
- 1:10 diluted by adding 0.5 ml of the 0.5 McFarland to 4.5 ml of MHB or saline.
- The 1:10 dilution of *Pseudomonas aeruginosa* spp. ATCC 25922was streaked a lawn to a Mueller Hinton agar plate and allowed to dry 3-5 minutes.
- A10 µg meropenem or susceptibility disk was placed in the centre of the test area.
- In a straight line, streak tested organism from the edge of the disk to the edge of the plate. Up to four organisms can be tested on the same plate with one drug.
- Incubated overnight at 350ºC ± 20º C in ambient air for 16-24 hours.

Interpretation/ Results

- At the intersection of the test organism and the *Pseudomonas aeruginosa* spp. 25922, inside the Carbapenem susceptibility disk inhibition region. Check the plate for a clover leaf-type indentation after 16-24 hours of incubation.
- A clover leaf-like indentation of the *Pseudomonas aeruginosa* spp.25922 is the MHT positive measure, which develops inside the disk diffusion zone along the growth streak of the test organism. A positive test indicates the development of carbapenemase by the microorganism studied. The test microorganism was able to inactivate carbapenem, which diffuses from the disk after putting the disc on the MHA, by generating carbapenemase. This makes the susceptible E carbapenem. To expand towards the disk, *Pseudomonas aeruginosa* spp. ATCC 25922.
- The MHT negative test showed no *Pseudomonas aeruginosa* spp.25922 growth. A long the growth streak of the test organism within the field of disc diffusion.
- Refer the CLSI Guidelines (Ml00) for recommendations on detection of carbapenemase production in *Pseudomonas aeruginosa* spp. that test susceptible to carbapenem. (Franco, 2010)

Expected values: A positive MHT indicates that this isolate was producing a carbapenemase. A negative MHT indicates that this isolate was not producing a carbapenemase.

Method limitations: The class of carbapenemase were not be determined by the results of the MHT. Some isolates show a slight indentation but do not produce carbapenmase.

Procedure notes: Up to four organisms can be tested on the same MHA plate with one drug. Two drugs with up to 4 organisms can be tested on a 150 mm Mueller Hinton agar plate.

Optimum Conditions for MBL Production

The optimum condition of MBL production is depends on growth of *E. coli.* The growth of *Pseudomonas aeruginosa* spp. majorly controls by following factors such as pH, temperature, turbidity, incubation time, media, salt type and concentration, moisture, availability of oxygen, refrigeration time and dehydration.

Antibiogram of *Pseudomonas aeruginosa* spp. isolates

Pseudomonas aeruginosa spp.isolates were studied for their antibiogram pattern by disc diffusion technique as described by Bauer, *et.al.,* (1966). Against a panel of 7 antibiotics. The antibiotic discs used were obtained from Ravi Diagnostic microbiology laboratory, Uttarakhand. Isolates were tested for against 7 commonly used antibiotics viz. Amoxicillin (AMX) 10µg, Azithromycin (AZM) 10µg, Amikacin (AMK) 10µg, Penicillin (PEN) 10µg, Oxacillin (OXS) 10 µg, Tetracycline (TET) 10µg and Ticarcillin (TIC) 10µg Table-3.3. Isolates were inoculated in nutrient broth and incubated at 37^{o}C for 24 hrs. Each broth culture was smeared on Muller-Hinton agar (Hi-Media) plates using a sterile cotton swab. Plates were allowed to dry for few minutes and antibiotic discs were placed on the agar surface and plates were incubated for 12-24 hrs at

37°C. The sensitivity or resistance of isolates for a particular antibiotic was determined by measuring the diameter of the zone of inhibition of growth with Antibiotic zone scale (Hi-Media). The results were interpreted as sensitive or resistant based on CLSI interpretive standards (CLSI- 2007). (Nordmann, P., *et. al.*, 2011)

RESULT

The total 60 clinical samples, 27 were urine samples, 33 were blood culture samples. Among 60 clinical samples, 36 isolates of *Pseudomonas aeruginosa* species were isolated.

Table- 1.2 *Pseudomonas aeruginosa* species from different Clinical Samples (n=100)

S. No.	Clinical Samples	No. of Samples	Positive for *Pseudomonas aeruginosa* spp.	
			Number	**%**
1.	Urine	25	15	62.96
2.	Blood	38	21	15.16
	Total Sample Collected	**63**	**36**	

Table- 1.3 Culture Positivity of Study Population (n=100)

Culture	Frequency	Percent
No growth	10	62
Growth	17	37
Total	**27**	**99**

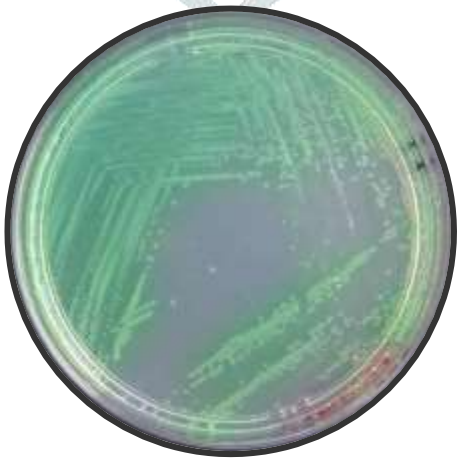


Plate- 1.1 *Pseudomonas aeruginosa* spp. in MacConkey Agar Plate

A very high percentage of *Pseudomonas aeruginosa* spp. [62.96%] was isolated from urine samples followed by pus samples, very low percentage of *Pseudomonas aeruginosa* spp. [15.16%] isolated from blood samples.

Sensitivity Pattern of *Pseudomonas aeruginosa* spp. to Amikacin

All the *Pseudomonas aeruginosa* spp. positive strains isolated from Urine were resistant (n=15) and all isolates from Pus were resistant (n=8) to Amikacin. 5 isolates of *Pseudomonas aeruginosa* spp. positive Blood sample, 3 were sensitive and 2 was resistant, 3 isolates of *Pseudomonas aeruginosa* spp. positive Sputum sample, 1 were sensitive and 2 was resistant to Amikacin.

Table- 1.4 Sensitivity Pattern of *Pseudomonas aeruginosa* spp. to Amikacin

S. No.	Clinical Sample	*Pseudomonas aeruginosa* spp.	
		Sensitive	Resistant
1.	Urine	0	15
2.	Blood	3	2
	Total	**3**	**17**
		20	

Sensitivity Pattern of *Pseudomonas aeruginosa* spp. to Amoxicillin/Clavulanate

It is observed that only 15 clinical samples were positive for *Pseudomonas aeruginosa* spp. growth from the total 25 clinical samples and remaining 10 samples, there was no growth of *Pseudomonas aeruginosa* spp. bacteria of the 15 isolates of *Pseudomonas aeruginosa* spp. positive Urine sample, 0 were sensitive and 15 were resistant; of the 38 isolates of *Pseudomonas aeruginosa* spp. positive Blood sample, 3 were sensitive and 2 were resistant to Amoxicillin/Clavulanate.

Table- 1.5 Sensitivity Pattern of *Pseudomonas aeruginosa* spp. to Amoxicillin/Clavulanate

S. No.	Clinical Sample	*Pseudomonas aeruginosa* spp.	
		Sensitive	Resistant
1.	Urine	6	4
2.	Blood	13	8
	Total	**19**	**12**
		31	

Sensitivity Pattern of *Pseudomonas aeruginosa* spp. to Cefuroxime

All the *Pseudomonas aeruginosa* spp. positive strains isolated from Sputum were resistant (n=4) to Cefuroxime. *Pseudomonas aeruginosa* spp. positive Urine sample, 6 were sensitive and 4 were resistant; 5 isolates of *Pseudomonas aeruginosa* spp. positive Blood sample, 13 was sensitive and 8 were resistant to Cefuroxime.

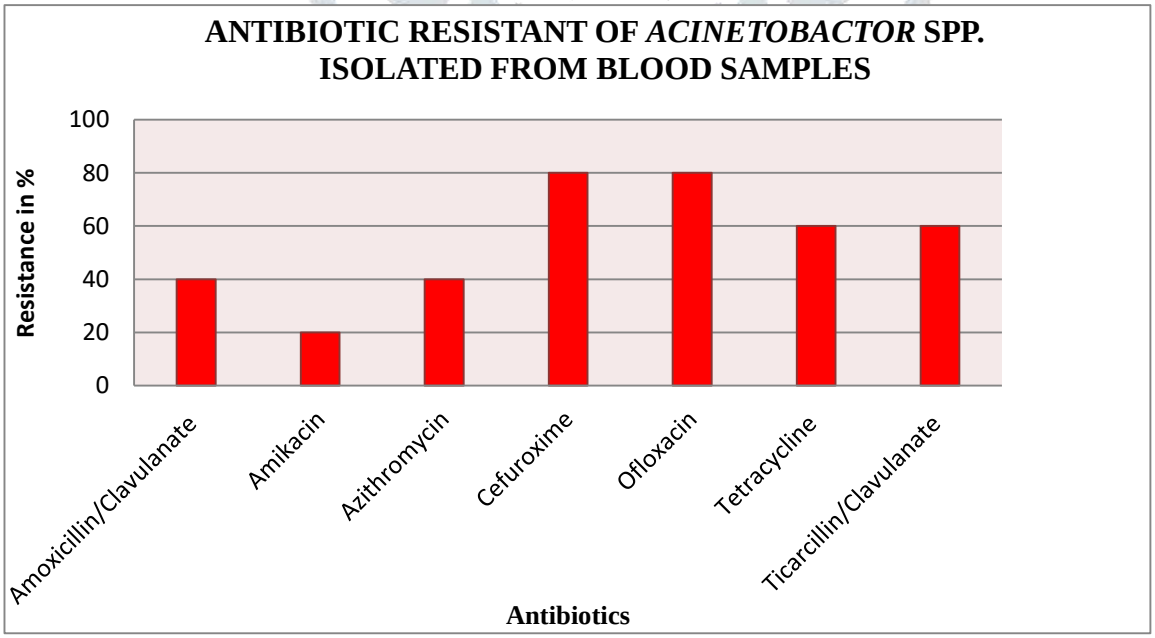
Table- 1.6 Sensitivity Pattern of *Pseudomonas aeruginosa* spp. to Cefuroxime

S. No.	Clinical Sample	*Pseudomonas aeruginosa* spp.	
		Sensitive	Resistant
1.	Urine	4	12
2.	Blood	1	4
	Total	**5**	**16**
		21	

Antibiotic Resistant of *Pseudomonas aeruginosa* spp. Isolated from Blood Sample

It was observed that *Acinetobactor* spp.isolates from Blood sample exhibited lowest resistance to Amikacin (20%) followed by Amoxicillin/Clavulanate and Azithromycin (40%), Tetracycline and Ticarcillin/Clavulanate (60%). Ofloxacin (64.71%). Wherever, highly resistant to both Cefuroxime and Ofloxacin (80%) (Figure 1.1).

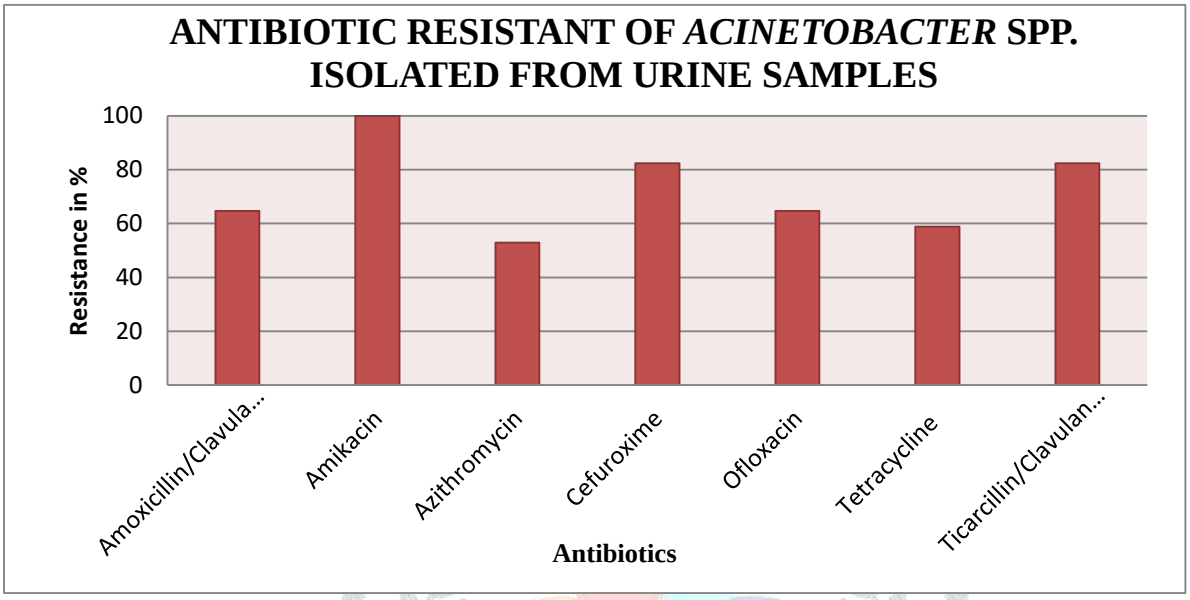
Figure- 1.1 Antibiotic Resistant of *Pseudomonas aeroginosa* Isolated from Blood Sample



Antibiotic Resistant of *Pseudomonas aeruginosa* spp. Isolated from Urine Samples

It was observed that *Acinetobactor* Spp. isolates from Urine sample exhibited lowest resistance to Azithromycin (52.94%) followed by Tetracycline (58.82%), Ofloxacin (64.71%), Amoxicillin/Clavulanate (64.70%), Cefuroxime and Ticarcillin/Clavulanate (82.35%). Wherever, highly resistant to Amikacin (100).

Figure- 1.2 Antibiotic Resistant of *Pseudomonas aeroginosa* Isolated from Urine



CONCLUSION

The total 60 clinical samples, 27 were urine samples, 33 were blood culture samples. Among 60 clinical samples, 36 isolates of *Pseudomonas aeruginosa* were isolated. A very high percentage of *Pseudomonas aeruginosa* spp. [62.96%] was isolated from urine samples followed by pus samples, very low percentage of *Pseudomonas aeruginosa* spp. [15.16%] isolated from blood samples. It is observed that only 15 clinical samples were positive for *Pseudomonas aeruginosa* spp. growth from the total 25 clinical samples and remaining 10 samples, there was no growth of *Pseudomonas aeruginosa* spp. bacteria of the 15 isolates of *Pseudomonas aeruginosa* spp. positive Urine sample, 0 were sensitive and 15 were resistant; of the 38 isolates of *Pseudomonas aeruginosa* spp. positive Blood sample, 3 were sensitive and 2 were resistant to Amoxicillin/Clavulanate.

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