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

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ABSTRACT

Acinetobacter spp.is oxidase negative, glucose, lactose, sucrose fermenting with an optimum growth pH of 6.0-7.0 and temperature of 37^0C. Some diarrheagenic *Acinetobacter* spp.strains have the ability to tolerate exposure to pH 2.0. Eosin Methylene Blue medium is suitable for isolation of *Acinetobacter* spp.from faeces and foods because of the ability to produce distinctive colonies having greenish metallic sheen and not produced by any other member of *Enterobacteriaceae.* The first β-lactamase was identified in *Acinetobacter* spp. prior to the release of penicillin for use in medical practice. The four major groups of β-lactams penicillin, cephalosporins, monobactams and carbapenems have a b-lactam ring which can be hydrolysed by β-lactamases resulting in microbiologically ineffective compounds. In Gram negative pathogens, b-lactamase production remains the most important contributing factor to β-lactam resistance. On the basis of mechanism of action, most common β-lactamases are divided into three major classes (A, C & D) depending on amino acid sequences. These enzymes act on many *penicillins, cephalosporins* and *monobactams.* Class B β-lactamases called as metallo beta lactamases (MBLs), act on *penicillins*, *cephalosporins* and *carbapenems* but not on monobactams.

Keywords: - Antibiotic resistance, β-lactamases Carbapenems, Motility, Physiochemical.

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 *Acinetobacter* spp. positive.

Koraei, *et.al* (2018) Cross-sectional study was performed on 726 different isolates of bacterial (*Enterobacteriaceae*) species. The samples were collected from different clinics including urine (n=461, 63.4%), blood (n=39, 5.4%), tracheal aspirate (n=60, 8.3%), wound (n=86, 11.9%), discharge (n=64, 8.8%) and abscess

(n=16, 2.2%) of hospitalized patients at two university-affiliated hospitals Golestan and Emam Khomeini in Ahvaz, southwest Iran for a period from June 2014 -July 2015. The clinical isolates were diagnosed as Enterobacteriaceae by standard biochemical tests and observed that from the total 726 clinical samples only 376 isolates (51.7%) identified as *Acinetobacter* spp. positive.

Mishra, *et. al.,* (2012) Collected 1162 specimens from lower respiratory tract (LRT) for culture and sensitivity test during June to November 2008. Specimens processed in this study were sputum (n=1081), Endotracheal tube secretion (n=61) and bronchial washing (n=20). Out of total 1081 sputum specimens, only 1039 specimens were further processed while the remaining 42 specimens were rejected as they implied oral contamination; mean 1120 samples were taken for further process. From the total 1120 samples, 454 samples of sputum, 41 samples of Endotracheal tube secretion and 2 samples of Bronchial washing were showed significant bacterial growth species.

Banu, (2011) Collected 200 samples from unhealthy patients for the bacterial isolates. The samples for sputum were collected at random. During the study period, the test samples were collected in a sterile specimen bottles (early in the morning).The patients were given specific instructions as to the collection of the samples. This is to avoid saliva but true sputum to get an accurate result.

After the testing of samples, 44 numbers of samples were found to be *Acinetobacter* spp. positive out of 200 samples. And also found that 64 numbers of samples were *Klebsiella pneumonia*, 65 samples of *Streptococcus pneumoniae* and 36 numbers of samples were *Staphylococcus aureus* positive. (Rasmussen, 1997)

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 *Acinetobacter* 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 *Acinetobacter* 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 *Acinetobacter* 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 *Acinetobacter* 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 *Acinetobacter* 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 *Acinetobacter* 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 *Acinetobacter* 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
- *Acinetobacter* 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 *Acinetobacter* 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 *Acinetobacter* 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 *Acinetobacter* 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 *Acinetobacter* 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, *Acinetobacter* spp. ATCC 25922.
- The MHT negative test showed no *Acinetobacter* 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 *Acinetobacter* 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 *Acinetobacter* 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 *Acinetobacter* spp. isolates

Acinetobacter 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 37ºC 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 *Acinetobacter* species were isolated.

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

S. No.	Clinical Samples	No. of Samples	Positive for *Acinetobacter* 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 *Acinetobacter* spp. in MacConkey Agar Plate

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

Sensitivity Pattern of *Acinetobacter* spp. to Amikacin

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

Table- 1.4 Sensitivity Pattern of *Acinetobacter* spp. to Amikacin

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

Sensitivity Pattern of *Acinetobacter* spp. to Amoxicillin/Clavulanate

It is observed that only 53 clinical samples were positive for *Acinetobacter* spp. growth from the total 100 clinical samples and remaining 47 samples, there was no growth of *Acinetobacter* spp. bacteria of the 15isolates of *Acinetobacter* spp.positive Urine sample, 6 were sensitive and 4were resistant; of the 21 isolates of *Acinetobacter* spp. positive Blood sample, 13were sensitive and 8 were resistant; of the 7 isolates of *Acinetobacter* spp. positive Sputum sample, 3 was sensitive and 4 were resistant; of the 10 isolates of *Acinetobacter* spp. positive Pus sample, 6 were sensitive and 4 were resistant to Amoxicillin/Clavulanate.

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

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

Sensitivity Pattern of *Acinetobacter* spp. to Cefuroxime

All the *Acinetobacter* spp. positive strains isolated from Sputum were resistant (n=4) to Cefuroxime. 17 isolates of *Acinetobacter* spp. positive Urine sample, 4 were sensitive and 13 were resistant; 5 isolates of *Acinetobacter* spp. positive Blood sample, 1 was sensitive and 4 were resistant; of the 9 isolates of *Acinetobacter* spp. positive Pus sample, 2 were sensitive and 7 were resistant to Cefuroxime.

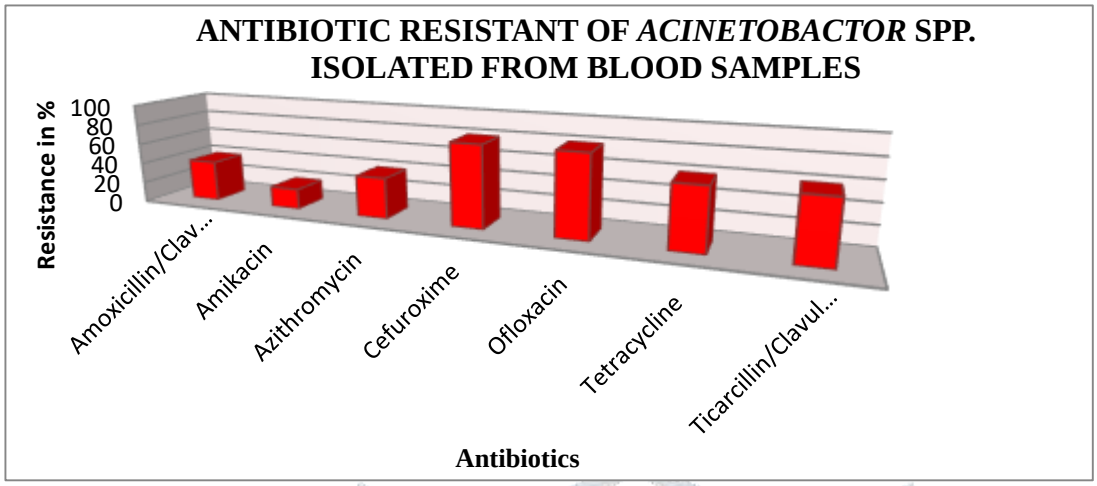
Table- 1.6 Sensitivity Pattern of *Acinetobacter* spp. to Cefuroxime

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

Antibiotic Resistant of *Acinetobacter* 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).

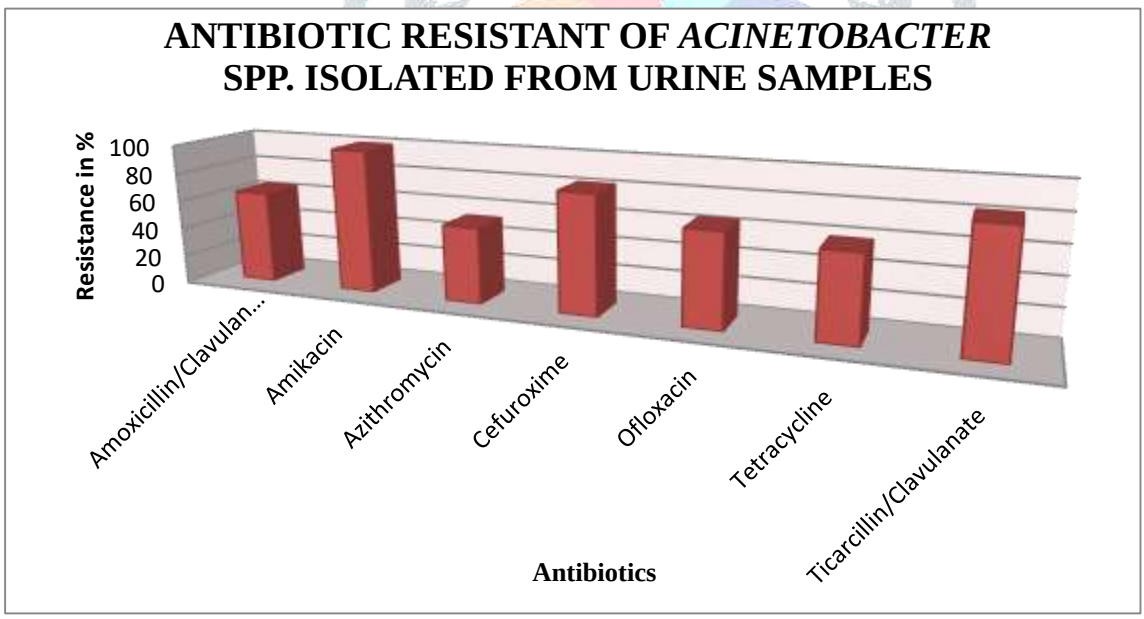
Figure- 1.1 Antibiotic Resistant of *Acinetobacter* spp. Isolated from Blood Sample



Antibiotic Resistant of *Acinetobacter* 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 *Acinetobacter* spp. Isolated from Urine Samples



CONCLUSION

The total 60 clinical samples, 27 were urine samples, 33 were blood culture samples. Among 60 clinical samples, 36 isolates of *Acinetobacter species* were isolated. A very high percentage of *Acinetobacter* spp. [62.96%] was isolated from urine samples followed by pus samples, very low percentage of *Acinetobacter* spp. [15.16%] isolated from blood samples. All the *Acinetobacter* spp. positive strains isolated from Urine were resistant (n=15) and all isolates from Pus were resistant (n=8) to Amikacin. 5 isolates of *Acinetobacter* spp.

positive Blood sample, 3 were sensitive and 2 was resistant, 3 isolates of *Acinetobacter* spp. positive Sputum sample, 1 were sensitive and 2 was resistant to Amikacin. 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).

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