Phenotypic and Genotypic detection of Metallo Beta Lactamase in Clinical isolates of *Acinetobacter* species

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ABSTRACT

Background; Carbapenem resistance medicated by Metallo beta lactamase (MBL) in *Acinetobacter* species is a global challenge due to its rapid spread and limited therapeutic option. Objective of the study to determine the prevalence of MBL in *Acinetobacter* species isolates in hospitalized patients by phenotypic and genotypic methods.

Material and method; non-duplicate isolates from ET, Blood, Sputum, Pus, Bal, and Csf etc. were included in this study. Isolate identification and antibiotic susceptibility testing of the isolates were done by standard microbiological methods according to CLSI guideline. The isolates which were resistance to carbapenem were tested by phenotypic (imipenem-EDTA combined disc test) and genotypic method for presence of common Metallo beta lactamase gene (blaIMP).

Result; A total of 369 *Acinetobacter* isolates were included in the study. Out of them 359(97.28%) were *Acinetobacter baumannii* and 10 (2.71%) were *Acinetobacter lwoffii*. Out of 369 isolates of Acinetobacter 338 were carbapenem resistance, among them 136(40.23%) were MBL positive and 202(59.76%) were MBL negative. The blaIMP gene was detected positively in 4(2.94%) isolates.

Conclusion; The most prevalent isolates in the investigation were *Acinetobacter baumannii*. The study reveals a higher prevalence of Metallo Beta Lactamase and the co-occurrence of blaIMP gene in carbapenem resistance Acinetobacter species.

Keywords: Acinetobacter, CLSI, Metallo Beta Lactamase, carbapenamase, combined disc test

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Introduction:

Acinetobacter are ubiquitous, free living, small aerobic gram negative cocco-bacilli that prefer moist environment and can be easily found in soil, water, and food, they are also most common organism in the hospital environment, they play a significant role in the colonization and infection of patients in hospital.[(1)] *Acinetobacter* species are emerging pathogen responsible for many infections such as bacteremia, nosocomial pneumonia, meningitis, surgical wound infection and urinary tract infection.[(2)]

Multidrug resistance *Acinetobacter baumannii* defined as an resistance to at least three or more different group such as penicillin's, cephalosporins, fluroquinolones and aminoglycosides has emerged and has been reported worldwide to significantly increase the morbidity, mortality and cost of treatment.[(3)] *Acinetobacter* species exhibit multidrug resistance through production of beta lactamase, alternation in outer membrane proteins and penicillin- binding proteins, and increase activity of efflux pumps.[(4)] Resistance to beta lactamase appear to be primarily caused by production of beta lactamase which include extended- spectrum-beta-lactamase (ESBL) Metallo Beta Lactamase (MBL) and Oxacillinase.[(5)] ESBL belong to Ambler class A beta lactamase, which shows resistance to third generation cephalosporins and inhibited by clavulanic acid, MBL belong to class B beta lactamase act on penicillin, cephalosporins, and carbapenems.[(6)] Ambler class C beta lactamase are amp C beta lactamase which confers resistance to cephalosporins in the oxyimino group, class D beta lactamase are OXA type beta lactamase have high hydrolytic activity against oxacillin and cloxacillin.[(7)]

The treatment of *Acinetobacter baumannii* infection have recently become challenging due to acquisition of resistance to various antibiotics by intrinsic and acquired mechanism.[(8)] Carbapenems are a class of antibiotics that are effective on both gram positive and negative bacteria, these-pathogen are resistance to penicillinase and cephalosporins. These carbapenems can be hydrolyzed by a class of beta

lactamase categorized as B subclass by Ambler classification known as Metallo beta lactamase (MBL). Metallo beta lactamase lead to numerous antibiotics resistance including penicillin, cephalosporins and carbapenems. [9] There are various MBL genes such as Imipenemase (IMP), Verona integron- encoded (VIM), Sno Paolo Metallo (SPM), New Delhi- Metallo (NDM), German Imipenemase (GIM). The most common gene are IMP, VIM and SPM of MBLs that have been detected in *Acinetobacter* species**.** [10] In present study, Imipenem EDTA Combined Disc test is used for detection of Metallo beta lactamase.

Material and Methods:

Study site and period

The present study was a cross-sectional study in conducted at a tertiary care hospital from North India.

Samples collection and processing

All clinical samples received (such as Sputum, Urine, ET, Pus, Blood, Bal, Csf etc. and other body fluid) in Microbiology lab. From patients admitted in various department of hospital has been included in this study. All the specimens were processed as per standard protocol technique using conventional microbiological culture method. All the specimen were cultured without delay on MacConkey agar, Blood agar and urine samples were inoculated on CLED agar and incubated aerobically at 37^0 C for 18-24 hours. After overnight incubation identification of isolates are based on the colony morphology, gram staining, catalase, oxidase and motility test and various biochemical tests like indole, citrate utilization test, urease test, and triple sugar iron agar test, oxidation fermentation test, Arginine dehydrolase test and hemolysis on blood agar. On gram stain *Acinetobacter* were seen as Gram Negative cocco-bacilli.

Antimicrobial susceptibility test (AST):

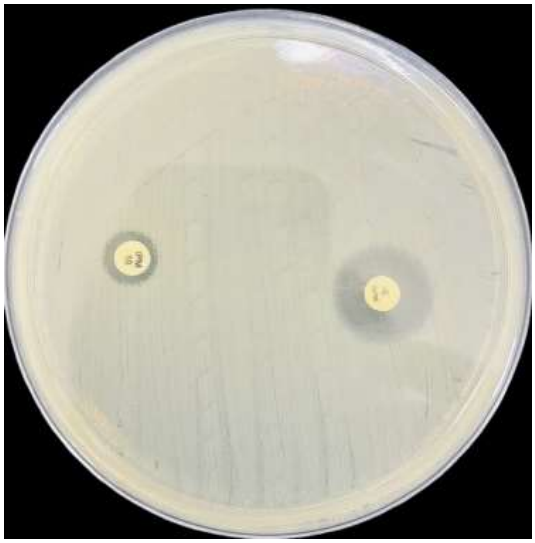
Antimicrobial susceptibility test (AST) Was performed using Muller Hinton agar (HiMedia laboratories, India) by Kirby Bauer disk diffusion method as per CLSI guideline. For disc diffusion testing, the antimicrobial disc used were as follow; Ampicillin-sulbactum, Ceftazidime, Cefepime, Ciprofloxacin, Levofloxacin, Gentamicin, Tobramycin, Imipenem, Meropenem, Amikacin, Piperacillin-Tazobactam, Trimethoprim-Sulfamethoxazole, Minocycline, Doxycycline, Cefotaxime, Ceftriaxone, Tigecycline, Colistin and for urine samples Fosfomycin, Nitrofurantoin, were used.

Phenotypic detection of Metallo beta Lactamase:

Imipenem- EDTA Combined Disc test-

The confirmation test for the detection of Metallo beta lactamase. Test and control organism inoculum was prepared according to 0.5 McFarland turbidity standard. The bacterial strains cultured on Muller Hinton agar plates, as per CLSI guideline. Imipenem 10 ug_disc placed 20 mm apart, center from an imipenem + EDTA disc containing 0.5 ul of 0.5 mg EDTA. Plates incubated at 37^0 C for 16-18 hours. On overnight incubation, an increase in zone size$\geq$ 7mm around the imipenem- EDTA disc compared to the imipenem alone was recorded positive for Metallo beta lactamase.

Figure 1: Imipenem EDTA Combined Disc Test



Genotypic detection of Metallo beta lactamase:

- PCR was used for screening of the IMP gene. QIAprep Spin Miniprep Kit was used for detection of gene by PCR. The primer used for IMP gene were;
- Forward: **GGAATAGAGTGGCTTAACTCTC**
- Reverse: **GGTTTAACAAAACAACCACC**

Using commercial DNA extraction kits, the samples DNA was extracted. PCR reaction was set up using a thermal cycler and included the following components: template DNA, forward and reverse primer specific to the target sequence. DNA polymerase, buffer solution containing salts and cofactors, and nucleotides (dNTPs). The PCR reaction underwent thermal cycling in the thermocycler. The cycling consisted of:

1. Denaturation- the double stranded DNA split into single strands when the reaction was heated to 94-95^{0}C for 30 second.
2. Annealing- to enable the primers to attach to their complementary sequence on the single- stranded DNA, the temperature was dropped to 60^{0}C for 30 second.
3. Extension- the primers were extended by the DNA polymerase by adding nucleotides to the template strand, creating new DNA strands, for 20-60 sec at a temperature of 72^{0}C.
   The following amplification, agarose gel electrophoresis was used to analysis a small portion of the PCR result.

Results:

A total of 369 non-duplicate *Acinetobacter* isolates were obtained from various clinical specimens. Of these, 359 were identified as *Acinetobacter baumannii* and 10 as *Acinetobacter lwoffii.*

Table 1: SPECIES OF *ACINETOBACTER* ISOLATED: (n =369)

S.NO.	SPECIES ISOLATED	NO. OF ISOLATES	PERCENTAGE %
1.	*Acinetobacter baumannii*	359	97.28%
2.	*Acinetobacter lwoffii*	10	2.71%
	Total	369	100%

Acinetobacter baumannii (97.28%) was the most common species isolated followed by *Acinetobacter lwoffii* (2.71%).

Table 2: DISTRIBUTION OF METALLO BETA LACTAMASE PRODUCING *ACINETOBACTER* SPECIES:

MBL Producer	136(40.23%)
MBL non-producer	202(59.76%)
Total	338

A total of 369 isolates of *Acinetobacter* species were tested for carbapenem resistance, out of which 338 isolates showed resistance to carbapenems. They were subjected to MBL production by combined disc test, out of which 136 (40.23%) showed Metallo Beta Lactamase activity by CDT.

Table 3: PREVALENCE OF METALLO BETA LACTAMASE PRODUCING *ACINETOBACTER* SPECIES:

Acinetobacter species	No of isolates	No of MBL producer	Percentage
Acinetobacter baumannii	359	133	37.04%
Acinetobacter lwoffii	10	3	30%
Total	369	136	36.85%

The overall percentage of MBL was 36.85%. the highest MBL production was seen in *Acinetobacter baumannii* 37.14% followed by *Acinetobacter lwoffii* 30%.

Table 4: DISTRIBUTION OF CLINICAL SAMPLES IN RELATION TO MBL STATUS IN TOTAL ISOLATES PRODUCING *ACINETOBACTER* SPECIES:

Specimens	MBL positive (136)	MBL negative (202)	Total (338)
ET	66(48.52%)	98(48.51%)	164
BLOOD	27(19.85%)	46(22.77%)	73
SPUTUM	13(9.55%)	16(7.92%)	29
PUS	10(7.35%)	15(7.42%)	25
BAL	5(3.67%)	8(3.96%)	13
OTHER	15(11.02%)	19(9.40%)	34

The highest MBL production was seen in ET 48.52% followed by blood 19.85%, sputum 9.55%, pus 7.35%, BAL 3.67% and other specimens such as csf, swab pleural fluid etc. was 11.02%.

TABLE 5: DISTRIBUTION OF GENES IN THE MBL PRODUCING ISOLATES:(136)

Isolates	blaIMP
Acinetobacter baumannii	3
Acinetobacter lwoffii	1
Total	4(2.94%)

Among 136 isolates of MBL, IMP gene was found in 4 (2.94%) isolates of Acinetobacter species.

Discussion:

Infection by multidrug resistant *Acinetobacter* species along with Metallo beta lactamase activities represent the therapeutic trouble in hospital environment especially in ICU.[11] *Acinetobacter* spp has become MDR, XDR, and PAN drug resistant due to widespread usage of antimicrobials worldwide in ICU. Carbapenems have been considered the final resort to treat multi-drug-resistant strain infections. The excessive usage of these antibiotics has now produced carbapenem-resistant strain. Production of Metallo beta lactamase (MBL) was found most common resistance mechanism in *Acinetobacter*.

In present study a total of 369 non-duplicate isolates of *Acinetobacter* species from various clinical specimens were included in this study, out of them *Acinetobacter baumannii* (97.28%) was the most common species isolated followed by *Acinetobacter lwoffii* (2.71%). The findings of present study were in accordance with the previous study Dhanapal Bindu *et al* (2025)[12], where they found *Acinetobacter baumannii* was predominant species accounting 103(95.3%) followed by *Acinetobacter lwoffii* 5(4.6%).

In our study 338 carbapenem resistance isolates of *Acinetobacter* species were further tested for Metallo Beta Lactamase by Imipenem-EDTA combined disc test. Out of 338, 136(40.23%) *Acinetobacter* isolates were identified as MBL positive and 202(59.76%) negatives for MBL test. A similar study done by Yadav Vikas Manasi *et al* (2024)[13] Out of 150 isolates maximum Metallo Beta-Lactamase (MBL) positive isolates were observed in *Acinetobacter baumannii* 75 (93%) followed by *Acinetobacter lwoffii* 4(3%) *Acinetobacter hemolyticus* 1(0.7%). A similar study done by N. Ashwin Chitrabanu *et al* 2021[14] concluded that Out of these 393 *Acinetobacter Baumannii* isolates 109 (27.7%) showed resistant to Imipenem. Out of these109 isolates, 65 (59.63) were positive for Metallo Beta lactamase production by double disc synergy test. Another similar study done by Kaur A et al 2018[15] in their study 44% isolates of *Acinetobacter baumannii* were positive for MBL detection. The rates of MBL positivity in *Acinetobacter* is highly and ranges from 9.3-70% at different geographical location in India. This variation could be due to difference in the antibiotic usage and judicious selection of antibiotics in their hospital settings.

In present study the incidence of IMP genes was 2.94%. IMP gene was shown to be mostly prevalent in *Acinetobacter baumannii* 3 followed by *Acinetobacter lwoffii* 1. A similar study done by Sakthi sree Jayakumar *et al* (2024)[16] in their study the IMP gene was less common, appearing in only 3(2%) isolates.

Conclusion:

The alarming increase in the frequency of MBLs producing *Acinetobacter* spp presents an emerging threat of complete resistance to all therapeutic drugs in practice, leaving the potentially toxic colistin and tigecycline as the only option among antibiotic. Therefore, it is essential to rapidly screen and detect MBLs producing *Acinetobacter* species to prevent the spread of drug resistance. Implementation of strict infection control practices and antimicrobial stewardship will help to prevent further dissemination.

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