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Detection of Metallo β-Lactamase in Acinetobacter Species Using Phenotypic Methods with their Antibiotic Resistance Profile in Tertiary Care Hospital

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Authors' contributions

This work was carried out in collaboration among all authors. Author MMR carried out the research works, analysed data, and prepared the manuscript. Author DN designed, conceptualised the study and analysed the data. Authors APK and SR contributed in analysis of the data. Authors MMR, DN and SR monitored the study. All authors read and approved the final manuscript.

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ABSTRACT

Background: *Acinetobacter species* are commonly found in nature, in water, and in soil, and also, and they have been isolated from humans and animals. In the hospital setting, *Acinetobacter species* has emerged as a major opportunistic pathogen, being able to colonize and develop

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infections in patients. In *Acinetobacter species,* β-lactamases and outer membrane alterations are likely to function together to impart resistance. Understanding the epidemiology, and resistance mechanism, and for infection management and preventing a potential global health crisis, techniques for identifying Carbapenem-Resistant-*Acinetobacter species* are essential.

Aim: To detect Metallo β-Lactamase (MBL) in *Acinetobacter species* with their antimicrobial susceptibility pattern.

Material and Methods: 100 *Acinetobacter species* isolates from various clinical samples such as blood, cerebrospinal fluid, sputum, bronchoalveolar lavage, pus, urine, wound swab, and body fluids were included in the study. Phenotypic tests such as, Disc Potentiation test & Modified Hodge test were used to detect the presence of Metallo β-lactamase in imipenem & meropenem-resistant isolates.

Results: The highest prevalence of *Acinetobacter* was found in blood (37%), followed by sputum (31%) and others [pus, urine, ascitic fluid, cerebrospinal fluid (CSF), bronchoalveolar Lavage (BAL), and pleural fluid]. A maximum number of *Acinetobacter* were isolated from MICU followed by medicine and surgery. The Modified Hodge test yielded the largest percentage of MBL-positive *Acinetobacter* isolates (67.26%) when compared to the Disc Potentiation test method (50.45%).

Conclusion: Early detection of Metallo β-lactamase production is essential for planning appropriate treatment according to the resistance mechanisms of the multi-drug resistant strains, as well as for efficient infection control procedures to prevent the spread of infection.

Keywords: Acinetobacter specie; metallo β-lactamase; antibiotic resistance; disc potentiation test (DPT); Modified Hodge Test (MHT).

1. INTRODUCTION

Acinetobacter species are non-fastidious, strict aerobe, gram-negative coccibacilli, oxidase-negative, catalase-positive, non-fermentative, and non-motile [1-3]. *Acinetobacter species* are commonly found in nature, in water, and in soil and also, and they have been isolated from animals. In humans, they are found to reside on the mucous membrane, skin, and also sometimes the membrane of the intestinal tract [3,4]. They are usually plumpy, and small, measuring 1.0-1.5 µm by 1.5-2.5 µm in diameter [5-8]. More than 50 species in the *Acinetobacter genus*, the *Acinetobacter baumanni complex* (*Acinetobacter nosocomialis, Acinetobacter pitti,* and *Acinetobacter baumanni*) are the most clinically important ones. But sometimes they develop into more coccoid form, usually present in pairs or long chains of variable duration [5]. It has led to many hospital outbreaks in the past years due to its ability to survive in the hospital environment and stay long on surfaces. In recent years, it has been labelled as a “red alert” human pathogen, expressing problems among the medical fraternity due to its wide range of antibiotic resistance [6].

In the hospital setting, *Acinetobacter baumannii* has emerged as a major opportunistic pathogen, being able to colonize and develop infections in patients with ventilator-associated pneumonia, secondary meningitis, urinary tract infection, septicemia, and other conditions in the intensive care unit (ICU). It also causes infections in other immune-compromised individuals, including burn patients [7].

Infections with *Acinetobacter baumanni*, particularly Carbapenem-Resistant *Acinetobacter baumannii* (CRAB), because of their high case rates, mortality, and morbidity, are of public health concern worldwide. According to the World Health Organization (WHO), carbapenemresistant *Acinetobacter baumannii* has high rates globally in the target list of antibiotic-resistant bacteria, as a vital priority pathogen to drive drug research and development [8]. Owing to the widespread use of β-lactam antimicrobials, bacterial resistance has increased and is now a significant threat to the continued use of antibiotic therapy [9]. The objective of this study is to enhance the clinical management of patients suffering from infections and detection of metallo β-lactamase which in turn will also provide us with relevant epidemiological details.

2. MATERIALS AND METHODS

This cross-sectional prospective study was conducted in the department of microbiology, MGM Medical college & hospital, Navi Mumbai, India from July 2019 to January 2021.

Sample size 100 *Acinetobacter species* isolates from various clinical samples such as blood,

cerebrospinal fluid, sputum, bronchoalveolar lavage, pus, urine, wound swab, and body fluids were included in the study. Samples received from age group below 18 years were excluded. Antibiotic Susceptibility testing of all *Acinetobacter species* isolated was carried out by the Modified Kirby Bauer Disc diffusion technique by using Mueller Hinton Agar (MHA) plates according to clinical and laboratory standard institute (CLSI) guidelines 2020 [10].

2.1 Test for Metallo β-lactamase Detection

2.1.1 Disc Potentiation Test (DPT) [11]

The standard suspension of test isolate was inoculated on Mueller Hinton Agar (MHA) plate. A 0.5M solution of EDTA was prepared by dissolving 186.1gm of disodium EDTA $2H_2O$ in 1000ml of distilled water and pH was adjusted to 8.0 by using NaOH. The mixture was sterilized by autoclaving. Two 10µg Meropenem discs and Imipenem discs were placed 20mm apart from center to center onto MHA plate. To one disc of Imipenem and Meropenem each, 5µl of 0.5M EDTA was added. The zone of inhibition of Meropenem and Meropenem/EDTA discs and Imipenem and Imipenem/EDTA discs were compared after 16-18 hours of incubation at 35^{o}C. An increase in the zone of inhibition at least by 7 mm with Meropenem/EDTA discs and Imipenem/EDTA disc than Meropenem and Imipenem alone respectively was considered as MBL positive [as shown in Fig. 1].

2.1.2 Modified Hodge Test (MHT) [11]

A 0.5 McFarland dilution of the *Escherichia coli* ATCC 25922 in 5 ml of broth or saline was prepared. In the Mueller Hinton agar (MHA) plate, 1:10 dilution was streaked as a lawn. In the center of the test region, a 10 g Imipenem disc was placed, the test organism was streaked in a straight line from the disc edge to the plate edge, and it was then kept in a bacteriological incubator at 35°C for 24-48 hours. The appearance of the enhanced ATCC *E. coli* 25922 growth along the test organism, which showed a cloverleaf-like indentation, which indicated a positive test, was used to detect the production of carbapenemase [as shown in Fig. 2].

3. RESULTS

A total 100 isolates of Acinetobacter species collected from various clinical samples of patients admitted and also out door patients (OPD) were included in the study. Clinical samples were from all the clinical areas of the hospital. Samples received from age group below 18 years were excluded from the study.

All isolates were non-duplicate and identified by conventional methods.

3.1 Demographic Distribution

Age and sex-wise distribution of 100 strains of Acinetobacter species in various clinical samples is shown in Table 1. The maximum number of Acinetobacter species were isolated from male patients (79%) in both age groups as compared to the female population (21%). The highest prevalence of Acinetobacter was found in Blood (37%), followed by Sputum (31%) and others (Pus, Urine, Ascetic Fluid, CSF, BAL and Pleural Fluid).

3.2 Antimicrobial Susceptibility Profile

All the 100 isolates of Acinetobacter species were subjected to antimicrobial susceptibility testing (AST) by using first line antibiotics to identify the multi-drug resistant strains. Table 2 shows the resistance pattern of Acinetobacter species, revealing that 88% of Acinetobacter species were resistant to Augmentin (AMC), followed by followed by Nitrofurantoin (82%), Tobramycin (68%), Cefotaxime, and Piperacillin (67%).

62 isolates of Acinetobacter species were found to be resistant to three or more antibiotic groups and considered as multi-drug resistant Acinetobacter species, which were further subjected to AST by using second line antibiotics as shown in Table 3. In the second-line antibiotics, *Acinetobacter species* were found to be highly resistant to Ticarcillin/ Clavulanic acid (61, 98.38%), followed by Piperacillin/Tazobactam (59, 95.16%), Meropenem (57, 91.19%) and Imipenem (56, 90.32%).

3.2.1 Phenotypic detection of metallo β-lactamase (MBL) production in acinetobacter species

The phenotypic detection of Metallo β-lactamase (MBL) was done on those isolates of Acinetobacter species which were resistant to Imipenem (56) and Meropenem (57). Disc

Potentiation test (DPT) and Modified Hodge test (MHT) were used for the phenotypic detection of MBL. Table 4 shows the proportion of MBL in imipenem and meropenem resistant strains of Acinetobacter species. The highest percentage of MBL-positive Acinetobacter isolates were given by the M.H.T (67.26%) method as compared to D.P.T (50.45%) method.

3.3 Result Summary

The study analyzed 100 non-duplicate Acinetobacter isolates from clinical samples of patients aged 18 and above. Most isolates were from male patients (79%) and were most commonly found in blood (37%) and sputum (31%) samples. Antimicrobial susceptibility testing revealed high resistance rates to first-line antibiotics, with 88% resistant to Augmentin and 82% to Nitrofurantoin. Additionally, 62 isolates were multi-drug resistant (MDR), showing significant resistance to second-line antibiotics such as Ticarcillin/Clavulanic acid (98.38%) and Piperacillin/Tazobactam (95.16%). Phenotypic detection of Metallo β-lactamase (MBL) among Imipenem and Meropenem-resistant strains

Table 1. Age-wise and sex-wise distribution of acinetobacter species in all the clinical samples

Samples	18-50 Age		51 & above		Isolates
	Male	Female	Male	Female	of *Acinetobacter*
Blood	12	10	12	3	37
Sputum	9	2	16	4	31
Pus	13	1	4	0	18
Urine	3	0	5	1	9
Ascitic Fluid	0	0	2	0	2
Cerebrospinal Fluid (CSF)	1	0	0	0	1
Pleural Fluid	0	0	1	0	1
Broncho alveolar Lavage (BAL)	1	0	0	0	1

Table 2. Antibiotics (first line antibiotics)

Antibiotic	Concentration (mcg)	Sensitive	Intermediate	Resistant
Amoxyclav (AMC)	20/ 10 mcg	10	2	88
Nitrofurantoin (NIT)	300 mcg	10	8	82
Tobramycin (TOB)	10 mcg	31	1	68
Cefotaxime (CTX)	30 mcg	30	3	67
Piperacillin (PI)	100 mcg	24	9	67
Amikacin (AK)	30 mcg	34	0	66
Ceftazidime (CAZ)	30 mcg	36	0	64
Gentamicin (GEN)	10 mcg	30	7	63
Trimethoprim- sulfamethoxazole (COT)	23.75/ 1.25 mcg	30	9	61
Ciprofloxacin (CIP)	5 mcg	34	10	56
Levofloxacin (LE)	5 mcg	64	0	36
Tetracycline (TE)	30 mcg	73	3	24

Table 3. Antibiotics (second line antibiotics)

Antibiotics	Concentration(mcg)	Sensitive	Intermediate	Resistant
Ticarcillin/ Clavulanic acid (TCC)	75/10 mcg	0	1	61
Piperacillin/ Tazobactam (PIT)	100/ 10 mcg	3	0	59
Meropenem (MRP)	10 mcg	3	2	57
Aztreonam (AT)	30 mcg	2	3	57
Imipenem (IPM)	10 mcg	5	1	56
Cefepime (CPM)	30 mcg	4	2	56
Tigecycline (TGC)	15 mcg	41	11	10
Levofloxacin (LE)	5 mcg	54	2	6
Colistin (CL)	25 mcg	58	0	4
Polymyxin B (PB)	300 units	60	0	2

indicated that the Modified Hodge Test (MHT) identified a higher proportion of MBL-positive isolates (Imipenem: 66.07%, Meropenem: 68.42%) compared to the Disc Potentiation Test (DPT) (both around 50%). This highlights a significant challenge in treating Acinetobacter infections due to the high prevalence of resistance to both first and second-line antibiotics and the presence of MBL producers.

4. DISCUSSION

During the study period of July 2019 to January 2021, 100 samples of *Acinetobacter species* was isolated from all the body fluids which were included in this study. Some of the studies and their findings are presented in Table 5.

Blood had the highest prevalence of *Acinetobacter species* accounting for 37%, followed by Sputum (31%), Pus (18%), Urine (9%), and Other body fluids (1%). Our study is comparable with the study conducted in 2010 in which the highest prevalence was also found in blood (47%) [12]. In controversy, in a study done by M. Hajjar et.al, the sputum sample had the highest prevalence (43%) [13].

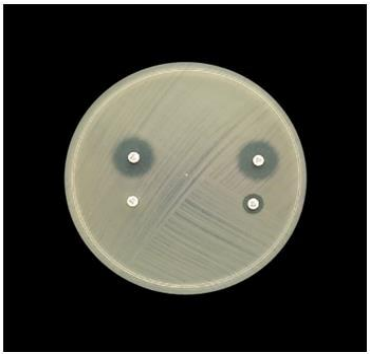

Fig. 1. Disc Potentiation Test (DPT) positive for Metallo β-Lactamase (MBL)

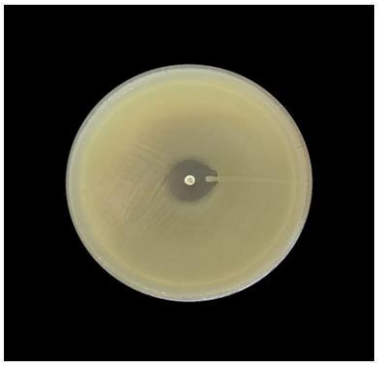

Fig. 2. Modified hodge test for Metallo β-Lactamase (MBL) production

In our study, when tested the antimicrobial susceptibility of the *Acinetobacter species* it was revealed that the *Acinetobacter* showed 88% resistance to Augmentin (AMC), which was followed by Tobramycin (68%). On the other hand, the *Acinetobacter species* showed the highest sensitivity to Tetracycline (TE) with a percentage of 68%. In the second-line antibiotics, the highest degree of resistance was found to be 98.38% for Ticarcillin/ Clavulanic acid (TCC) and Piperacillin/ Tazobactam (PIT). On the other hand, the highest sensitivity was found against Polymyxin B (PB) with 96.77% followed by 93.5% to Colistin (CL), 87.09% to Levofloxacin (LE), and 66.12% to Tigecycline (TGC). Another study done by Dr. S. Das, et.al, [14] presented the Antimicrobial Susceptibility Pattern which showed 100% susceptibility to Colistin, followed by 75% to Aztreonam and 65% sensitivity to Imipenem and Meropenem. Along with this, it was also found that the lowest sensitivity was towards Ceftazidime (21.7%). Our present study is comparable with the study done by M. Hajjar, et.al [13] in which according to Antibiotic Susceptibility Testing, 95% of *Acinetobacter species* were resistant to Cefoxitin, 87% were resistant to Ciprofloxacin, 86% resistant to Trimethoprim-sulfamethoxazole and Piperacillin-tazobactam, 83% were resistant to Cefotaxime, 81% to Ceftazidime and 80% to Gentamicin. Also, 78% of the isolates of *Acinetobacter species* were resistant to Imipenem and 84% to Meropenem. There was only 1 isolate that was resistant to Colistin. In another study done in the year 2018 by A. Kaur, et.al, [15], most of the antibiotics examined demonstrated high levels of resistance in *Acinetobacter baumannii isolates. Acinetobacter baumannii strains* were found to be resistant to Ceftazidime in 96.6% of cases, Cefepime in 94.8% of cases, Imipenem in 60.3% of cases and Meropenem in 68.1% of cases. However, Polymyxin B has a susceptibility of 96.5% and Colistin has a susceptibility of 97.4%.

In the present study, two age groups were considered to analyze their distribution. In this study, it was found that the species of *Acinetobacter* were widely distributed in the age group of 1850 (52%) as compared to 51 and above (48%). The other study done in the year 2020 by S.K. Yadav, et.al, [16], included various age groups in their study. In their study, the highest distribution of *Acinetobacter species* was in the age group of 16-32 years (23.6%) followed by ≥ 65 years (22.4%), ≤ 15 years (19.8%), 33-48 years (18.7%) and 49-64 years (15.5%). The other study done in the year 2018

Table 4. Metallo β-lactamase production by different methods among the imipenem and meropenem resistant *Acinetobacter spp*

Antibiotics	Disc Potentiation Test (DPT)		Modified Hodge Test (MHT)	
	Positive	Negative	Positive	Negative
Imipenem (n=56)	28	28	37	19
Meropenem (n=57)	29	28	39	18

Table 5. Various studies and their sample-wise distribution

Sample	Present Study	S.K. Yadav, et.al [16]	Dr. S Das, et.al [14]	M Hajjar, et.al [13]	A.Rezaei,et.al, [20]	F Sahcher aghi, et al [12]
Blood	37%	6.2%	13.3%	5%	3%	47%
Sputum	31%	-	3%	43%	3%	10%
Pus	18%	27.3%	38.3%	21%	9%	12%
Urine	9%	6.8%	15%	10%	-	7%
Other Body Fluids	5%		-	19%	-	
Catheter Tips	-		-	2%	1.2%	
Medical Devices	-		20%	-	-	

by V. Rebic, et.al, [17], studied the distribution of *Acinetobacter species* in three age groups. The ratio of isolates was higher in the over 60 years age group (p= 0.763). In the present study, it was discovered that the isolates of *Acinetobacter species* were more in males (79%) as compared to females (21%). *Acinetobacter* infections were more frequent in males (54.20 %) as compared to females (45.80 %). In the present study, the growth of *Acinetobacter* isolates in blood was more in the age group of 18-50 with males (12) and females (10), which was followed by sputum in which the highest number of isolates were found from the age group of 51 and above with males (16) and females (4). In the pus sample the maximum number of *Acinetobacter* was isolated from the age group of 18-50 in which males were 13 and female was 1. In the urine sample, the highest isolates were found in the age group of 51 and above with males (5) and female (1).

The present study was conducted on 100 isolates of *Acinetobacter species*, to detect the prevalence of MBL. The production of MBL was carried out on those isolates that were resistant to Meropenem (57) and Imipenem (56). MBL was detected with the help of 2 methods- Disc Potentiation Test (DPT) and Modified Hodge Test (MHT). 56 Imipenem resistant *Acinetobacter* isolates showed 50% positivity by DPT and 66.07% by MHT. Whereas, out of 57 Meropenem-resistant *Acinetobacter species* isolates, 50.87% were positive by DPT and 68.42% by MHT. In a study done by Zahra Moulana, et.al [18], in the year 2020, the use of DDST was one of the more effective techniques for the detection of Ambler class B MBL production with a high rate of positivity, according to the results of several studies. The results from the MHT showed that 84% of *A.baumannii* isolates were carbapenemase producers. Besides that, their study showed the lowest positivity rate (30%) by the DDST method. Our study is comparable with the study done in the year 2019 by Mittal N, et.al. [19] in which 292 (76.8%) isolates were likely MBL producers with only 72 (19%) confirmed as MBL producers.

5. CONCLUSIONS

The maximum number of *Acinetobacter species* was isolated from the age group 18-50 years as compared to the age group of 50 & above. The present study showed that Males had predominance over Females (4:1) in both age groups in all the samples. According to the present study, the *Acinetobacter species* showed the highest percentage of resistance to Augmentin (AMC) (88%), which was followed by Nitrofurantoin (NIT) (82%). On the other hand, the *Acinetobacter species* showed the highest

susceptibility to Tetracycline (TE) (73%). In the second line of antibiotics, the highest degree of resistance was found to be for both Ticarcillin/ Clavulanic acid (TCC) and Piperacillin/ Tazobactam (PIT) i.e., 98.38%. On the other hand, the highest susceptibility was found against Polymyxin B (PB) (96.77%) followed by Colistin (CL) (93.5%), Levofloxacin (LE) (87.09%), and Tigecycline (TGC) (66.12%). The highest percentage of MBL-positive *Acinetobacter* isolates were given by the M.H.T (67.26%) method as compared to D.P.T (50.45%) method. The potential spread of these multi-drug resistant strains may be stopped by the early detection of MBL-producing Acinetobacter species. To find MBL producers, active monitoring is required. In district health laboratories or other locations lacking access to molecular diagnostic tools, the phenotypic tests described in the current research can be used to quickly identify these types of resistance.

6. LIMITATIONS

The lack of PCR analysis for the verification of phenotypical techniques constitutes one of the study's limitations. It was a uni-center study so our findings cannot be generalized to the entire region.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

HIGHLIGHTS

- To identify and characterize the proportion and distribution of MBL in Imipenem and Meropenem Resistant isolates within the clinical isolates of Acinetobacter.
- To analyse the phenotypic characteristics of MBL producing Acinetobacter isolates, including their antimicrobial resistance pattern.

CONSENT AND ETHICAL APPROVAL

The approval of the Institutional Ethics Committee (N-EC/2019/SC/07/97) IRB name (Dr. Ipsheeta Ray & Dr. Sarita Sahani) and the written consent was obtained from all the patients.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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