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Research Article

Antibiotic Resistance of
Acinetobacter Species

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Isolation and Speciation of *Acinetobacter* Species with Special Reference to Antibiotic Resistance in Tertiary Care Hospital

Manasi Vikas Yadav[1], Dr. G. S. Karande[1]*, Dr. S.R. Patil[1], Dr. R.V. Shinde[1], Dr. S.K. Pawar[1], Dr. H.V. Patil[1], Dr. P. M. Mane[1] and Shanu Sharma[1].

[1]*Department of Microbiology, Krishna Institute of Medical Sciences, (Deemed to Be University), Karad, Malkapur, Karad - 415539 (District Satara), Maharashtra, India.*

[1]**, Professor, Department of Microbiology, Krishna Institute of Medical Sciences, Karad.*

Abstract: *Acinetobacter* can survive longer in the environment and is also known for developing resistance against agents like disinfectants and nutritional deprivation. Very restricted information about *Acinetobacter* is available because of their confused taxonomic status. The isolation and identification of resistance pattern help in the selection and search for new antibiotics, reducing the morbidity and mortality of patients. The present study was conducted to find the different types of resistant mechanisms in *Acinetobacter* species and their antimicrobial susceptibility pattern from various clinical samples. *Gram's stain and biochemical reactions identified Acinetobacter* isolated from various clinical samples. Antibiotic susceptibility testing was done, and their resistant pattern was observed. Phenotypic methods for drug resistance were carried out -Detection of Extended Spectrum Beta Lactamase (ESBL) by Double disc synergy test (DDST), detection of Metallo Beta Lactamase (MBL) by Imipenem with EDTA, and detection of Carbapenemase production by Modified Hodge test (MHT). The results showed that out of 150 isolates of *Acinetobacter* species, *Acinetobacter baumannii* 138 (92%) was the most common species isolated, followed by *Acinetobacter lwoffii* 10 (7%) and *Acinetobacter hemolytic* 2 (1%). Of these, 22 (15%) isolates showed ESBL production by Double disc synergy test, 80 (53%) isolates were MBL producers, and 11 (7%) were Carbapenemase producers by Modified Hodge test. The study observed that ESBL production in *Acinetobacter* was 22(15%) and MBL 80(53%). It demonstrated that most of the *Acinetobacter* isolated were found to be Multi-Drug Resistant (95%). It brings out the need for active surveillance combined to eradicate the curtail of this organism in hospital settings.

Keywords: *Acinetobacter*, ESBL, MBL, AST, MHT, MDR.

*Corresponding Author

Dr. G. S. Karande , Professor, Department of Microbiology, Krishna Institute of Medical Sciences, Karad.

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1.   INTRODUCTION

Acinetobacter species are Gram-negative, strictly aerobic, non-fastidious, non-fermenting, encapsulated coccobacillus that mainly causes nosocomial infections.[1] *Acinetobacter* is a heterogeneous group of organisms, typically free-living saprophytes found almost everywhere and commonly distributed in the environment. However, different species of the genus are generally associated with various habitats, e.g., soil, water, sewage, human, foods, and animals.[2] They are isolated as commensals from the skin and throat.[3] *Acinetobacter* has emerged as one of the most troublesome pathogen classes in healthcare-associated infections like hospital-acquired pneumonia, community-acquired pneumonia, bacteremia, trauma, wound infection, urinary tract infection, meningitis and other manifestations of endocarditis, peritonitis, ophthalmitis.[4] Genus *Acinetobacter* comprises more than 50 validly named species, among which are *A. baumannii* group, *A. lwoffii, A. johnsonii, A. junii, and A. hemolytic*. The presence of a polysaccharide capsule formed of L-rhamnose, D-glucose, D-glucuronic acid, and D-mannose,[5] which probably renders the surface of strains more hydrophilic, although hydrophobicity may be higher in *Acinetobacter* strains isolated from catheters or tracheal devices.[6] The *Acinetobacter* infection is the most recent of all bloodstream infections occurring during hospitalization. It is the 10th common etiologic agent responsible for nosocomial bloodstream infections in the United States during 1995-2002, accounting for 1.3% of mono-microbial bloodstream infections.[7] Hospital-acquired pneumonia is still the most common infection caused by *Acinetobacter. Acinetobacter* in the community commonly causes meningitis. [7] secondary meningitis is predominant due to the Acinetobacter infection, although primary meningitis also has been reported during neurological procedures or head trauma.[8] It is a microorganism, characterized by the rapid development of resistance to most antimicrobials. It has appeared as a pathogen frequently incriminated in lower respiratory tract infections in critically ill patients. The biochemical tests are carried out to speciate the *Acinetobacter* species. The study of antibiotic resistance is necessary to help treat infections.[9] Due to long-term evolutionary exposure to soil organisms that produce antibiotics, *Acinetobacter sp.* can develop antibiotic resistance extremely rapidly. It contrasts with other clinical bacteria, which require greater time to acquire resistance, usually in response to therapeutic strategies. Conjugation, plasmids, and transposons (in conjunction with integrons) are important in transferring resistance determinants between different strains.[10] They are innately resistant to many antibiotics and are known to produce extended-spectrum Beta-lactamases and Metallo-Beta-lactamases. The carbapenems are Beta-lactam antimicrobial agents with an exceptionally broad-spectrum activity. *A. baumannii* infections are often difficult to eradicate due to high-level resistance to many antibiotics due to intrinsic and acquired mechanisms.[9] *Acinetobacter baumannii* production of Metallo-beta-lactamase (MBL) has become a worldwide therapeutic concern.[11] The present study was done to investigate the prevalence, bacteriological profile, and antimicrobial susceptibility pattern of *Acinetobacter* species isolated from various clinical samples with an objective to look for different types of resistant mechanisms which will provide valuable information and opportunity to other scientists in order to uncover the precise drug molecule to fight against infections caused due to *Acinetobacter* and standardize the living conditions. It is generally agreed that *A.*

baumannii is the most medically significant *Acinetobacter spp.* The clinical impact of *Acinetobacter* was increasing morbidity or mortality, and their infections are responsible for the increase in patient mortality in critically ill patients.[12] It is considered a low virulence organism except when isolated in critically ill or immunocompromised patients. These organisms are most often associated with nosocomial rather than community-acquired infections.[13,14] The ability of *A. baumannii* to develop multidrug resistance and to persist in harsh environmental conditions makes infections by *Acinetobacter* very dangerous, especially in individuals who have recently undergone major surgery, had malignant diseases or burnt or immunosuppressed patients such as the elderly, neonates with low birth weights, and patients with prolonged illnesses.[12] Patients with mechanical ventilation, particularly of prolonged duration, longer hospital or ICU stay, and a greater degree of exposure to infected or colonized patients in the neighboring hospital environment, have an increased risk for the acquisition of multidrug-resistant outbreak strains.[15]

2.   METHODOLOGY

"Isolation and Speciation of *Acinetobacter* Species with Special Reference to Antibiotic Resistance in Tertiary Care Hospital" was conducted in the Microbiology laboratory at Krishna Institute of Medical Sciences and Krishna Hospital and Medical Research Centre, Karad.

2.1.   Study Setting

The study was undertaken in the Department of Microbiology, Krishna Institute of Medical Sciences, deemed to Be University, Karad, India.

2.2.   Study Design

Observational cross-sectional study

2.3.   Study Period

November 2020 to November 2022

2.4.   Inclusion criteria

Isolates of *Acinetobacter* spp. from all clinical samples received in the laboratory were included. In addition, patients of both sexes were included.

2.5.   Exclusion criteria

Isolates of *Acinetobacter* species from the same patients and specimens were excluded from the study to avoid duplication of isolates.

2.6.   Statistical analysis

Data were filled in the MS Excel Software. Then, analyzed results were expressed as percentage and p values by Chi-square test using Graph Pad Instant software. If the probability is less than 0.05, the association or difference is said to be significant.

2.7.   Specimen collection

Clinical samples including pus, sputum, urine, ETT secretions,

blood, body fluids, wound swab, CSF, catheters, and various prosthetic devices from both gender and all groups of patients included. Samples were collected aseptically in sterile and appropriate containers storage and transportation in cold conditions till processing, except blood culture bottles.

2.8. Sample Processing

For primary identification of the *Acinetobacter* based on their microscopic observation, firstly, a clean, grease-free slide was taken. Upon that, a drop of normal saline was taken under aseptic condition. A small colony of bacteria was picked, and a thin, uniform smear was prepared. The smear was heat fixed. The heat-fixed smear was stained by the Gram stain technique and examined under the oil immersion objective of the light microscope. In Gram stain, Gram-negative bacilli were observed. The isolates were identified based on colony morphology on agar and Gram stain of the smear made from the colonies. Oxidase, catalase, and biochemical reactions were performed to identify colonies. All collected specimens were inoculated on Nutrient, MacConkey, blood, and chocolate agar and incubated at 37˚C for 24 hours. All clinical specimens received in the laboratory were inoculated on Nutrient agar, Cysteine lactose electrolyte deficient (CLED) agar, MacConkey agar, Blood agar, and Chocolate agar. The biochemical tests were performed according to the standard operating procedure mentioned in Mackie & McCartney Practical Medical Microbiology 14th edition.[16]

2.9. Antibiogram

Antimicrobial susceptibility testing of isolates was performed on Mueller Hinton agar using the Kirby-Bauer disc diffusion method. Four to five colonies of the same morphology were selected from an agar culture plate. With a sterile bacteriological loop, the growth was inoculated into a broth medium which was incubated for 3 to 5 hours to achieve a turbid suspension. It was compared with 0.5 McFarland standards. A swab was submerged in bacterial suspension and was inoculated on a Mueller Hinton agar plate. The surface of the plate was swabbed in three directions so that there was an even and complete distribution of the Inoculum. Within 15 minutes of inoculation, antibiotic discs were applied using sterile forceps. The plates were incubated at 37˚c for 24 hrs., after which the zone of inhibition was measured by using a zone measuring scale, and interpretation was made as per the CLSI guidelines.[17,18]

2.10. Detection of resistance mechanism

- **Detection of Extended Spectrum of Beta-Lactamase (ESBL) production by Double Disc Synergy test (DDST)**

Gram-negative bacilli isolated was suspected to be an ESBL producer if it was resistant to Aztreonam (30 µg), Cefotaxime (30 µg), Cefodoxime (10 µg), Ceftazidime (30 µg), and Ceftriaxone (30 µg). The Inoculum of test and control organisms was prepared and matched with turbidity 0.5 McFarland standard. ESBL production was tested by the Double Disc Synergy Test (DDST), using a disk of ceftazidime + clavulanic acid and ceftazidime (cephalosporin). The bacterial strains were cultured on Mueller Hinton agar plates per CLSI guidelines. A disc that contained ceftazidime + clavulanic acid (30µg+10µg) was placed on the plate at a distance of 25mm from that of ceftazidime (30µg) and allowed to diffuse at room temperature (for 1hr). The plate was incubated for 18-24 hr (at 35˚C). An increase greater or equal to 5 mm in the inhibition diameter of the ceftazidime disc was applied after the pre-diffusion of ceftazidime + clavulanic acid compared with the ceftazidime disc considered as positive for ESBL production.[19]

- **Detection of Metallo Beta Lactamase (MBL) by Imipenem-EDTA combined disc diffusion test**

If the increase in inhibition zone with Imipenem - EDTA disc is greater than or equal to 7 mm than the Imipenem (10 µg) alone, it is interpreted as an MBL producer.[10]

- **Detection of Carbapenemase production by Modified Hodge test**

A distorted zone of inhibition or clover leaf-like indentation, at the intersection of the test organism and the *E. coli* ATCC 25922, within the zone of Meropenem susceptibility disc, was interpreted as positive for Carbapenemase by Modified Hodge Test.[20]

3. OBSERVATION AND RESULTS

Table 1: Age and gender-wise distribution of *Acinetobacter*				
Age group	Male (n)%	Female (n)%	Total (n)%	Percentage %
0-20	8 (5)	6 (4)	14	9
21-40	28 (19)	18 (12)	46	31
41-60	42 (28)	15 (10)	57	38
> 60	22 (15)	11 (7)	33	22
Total (n)	100 (67)	50 (33)	150	100

X^2= 0.5993, p-value = 0.8966, Not significant

Age and gender-wise distribution of *Acinetobacter* isolated. The isolates in the age group of 0- 20 years were 14(9%), followed by the age group 21-40 years 46(31%), 41-60 years 57(38%), >60 years 33(22%) respectively. (Table No.1)

Table 2: Distribution of the *Acinetobacter* species isolated from various clinical specimens		
Specimens	Total	Percentage %
Pus	38	25
Tracheal aspirate	36	24

Urine	30	20
Sputum	16	11
Blood	16	11
Body fluids	12	8
CSF	2	1
Total	150	100

Sample-wise distribution of *Acinetobacter species*. The majority of the isolates were from pus 38 (25%), followed by tracheal aspirate 36 (24%), urine 30 (20%), sputum 16 (11%), blood 16 (11%), body fluids 12 (8%), CSF 2 (1%). (Table No.2)

Table 3: Location-wise isolation of *Acinetobacter* species		
Location	**Isolation**	**Percentage%**
Indoor	139	93
Outdoor	11	7
Total	150	100

Location-wise isolation of *Acinetobacter* species from patient's samples. In the present study, a maximum number of isolates were from Indoor patients, 139 (93%), then Outdoor 11 (7%). (Table No.3)

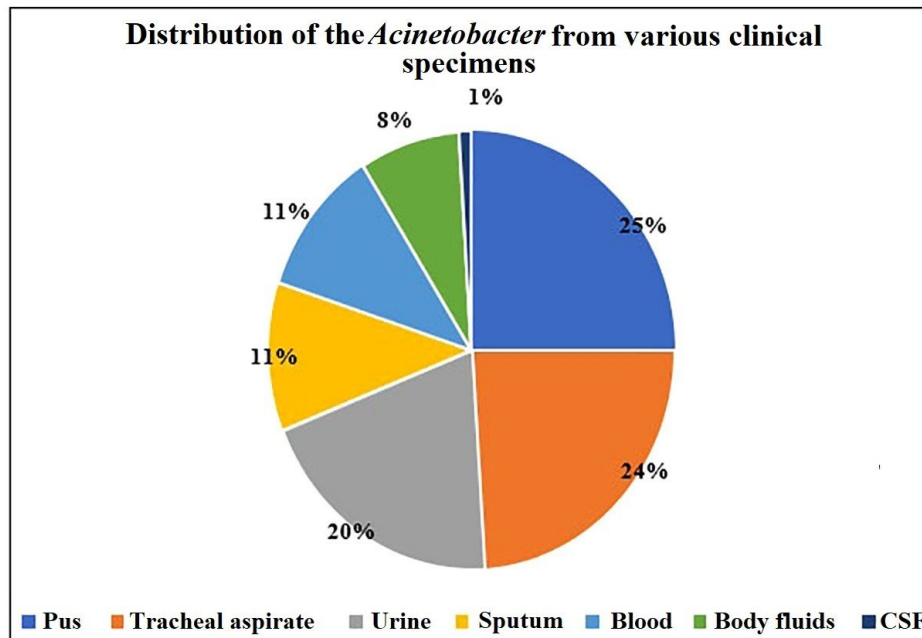| Table 4: Antibiotic susceptibility pattern of *Acinetobacter* species isolated from various clinical samples				
	Sensitive		**Resistant**	
Antibiotic	**No. of isolates**	**Percentage%**	**No. of isolates**	**Percentage %**
Amikacin	43	29	107	73.3
Ciprofloxacin	32	21.3	118	79
Cefepime	26	17.3	124	83
Piperacillin	32	21.3	118	79
Imipenem	44	29.3	106	71
Meropenem	38	25.3	112	75
Gentamicin	38	25.3	112	75
Levofloxacin	42	28	108	72
Tigecycline	106	71	44	29.3
Colistin	113	75.3	37	25
Co-trimoxazole	46	31	104	69.3
Nalidixic acid	22	15	128	85.3
Ampicillin	20	13.3	130	87
Ceftazidime	24	16	126	84

$$X^2 = 342.55, \text{ p value} = < 0.0001, \text{ significant}$$

The different resistance pattern of bacterial isolates was observed against antimicrobial agents. Maximum sensitivity to Colistin 113(75.3%) was showed by *Acinetobacter* species, followed by Tigecycline 106(71%), whereas, maximum resistance was to Ampicillin 130(87%), followed by Nalidixic acid 128(85.3%), Ceftazidime 126(84%). (Table No.4)
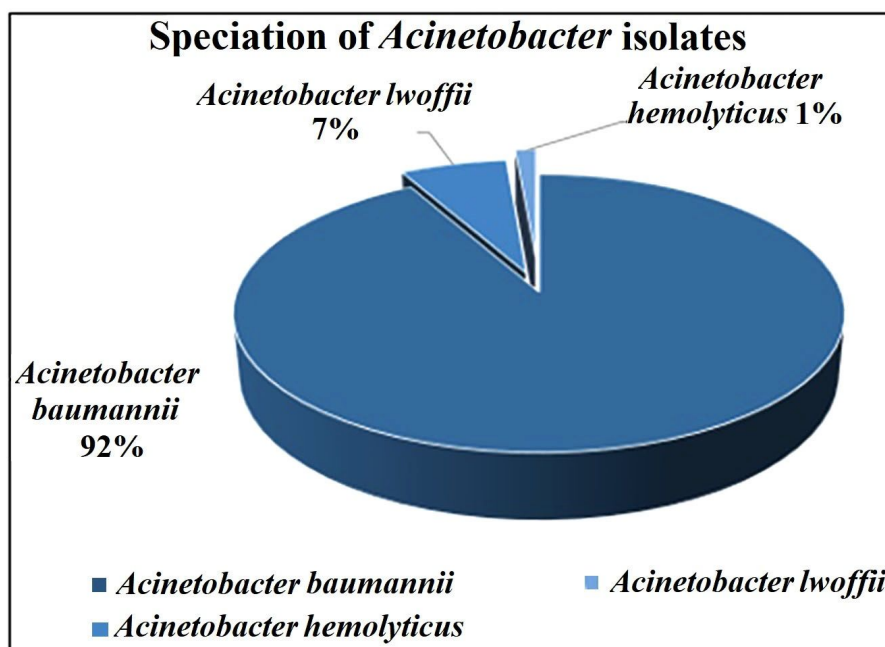
Table 5: Detection of Carbapenemase production among the *Acinetobacter* isolates by Modified Hodge Test (MHT)		
Test	**Number (n)**	**Percentage (%)**
MHT Positive	11	7
MHT Negative	139	93
Total	150	100

Out of 150 isolates, 11 (7%) were Modified Hodge test positive, and 139 (93%) were negative. (Table No.5)

Fig 1: Distribution of the *Acinetobacter* from various clinical specimens

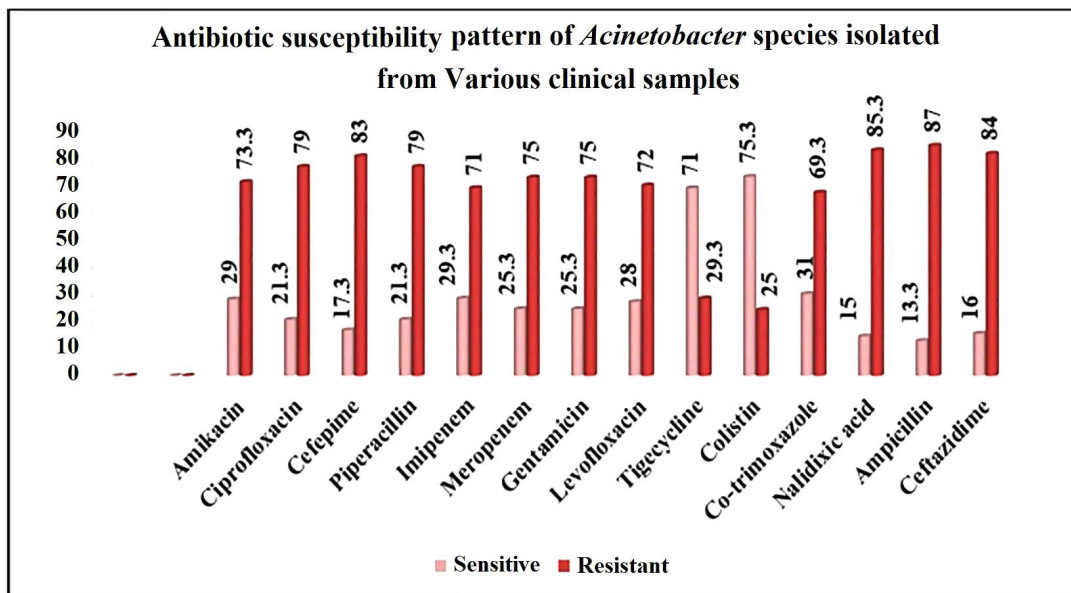**Fig 2 : Speciation of *Acinetobacter* isolates**

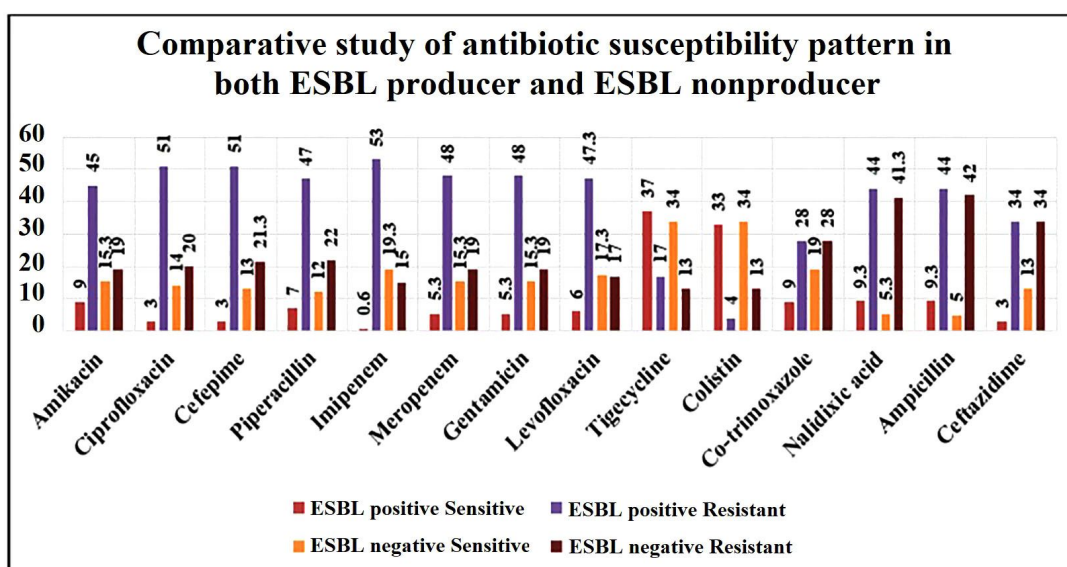**Fig 3: Antibiotic susceptibility pattern of Acinetobacter species isolated from various clinical sample**

Fig 4: Comparative study of antibiotic susceptibility pattern in both ESBL producer and ESBL non producer
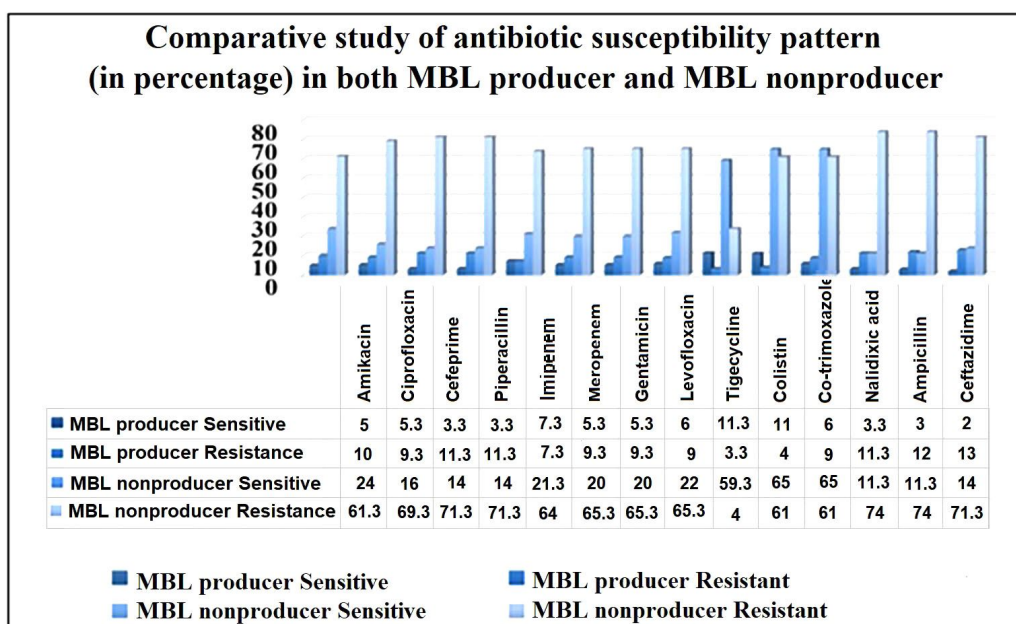

	Amikacin	Ciprofloxacin	Cefepime	Piperacillin	Imipenem	Meropenem	Gentamicin	Levofloxacin	Tigecycline	Colistin	Co-trimoxazole	Nalidixic acid	Ampicillin	Ceftazidime
MBL producer Sensitive	5	5.3	3.3	3.3	7.3	5.3	5.3	6	11.3	11	6	3.3	3	2
MBL producer Resistance	10	9.3	11.3	11.3	7.3	9.3	9.3	9	3.3	4	9	11.3	12	13
MBL nonproducer Sensitive	24	16	14	14	21.3	20	20	22	59.3	65	65	11.3	11.3	14
MBL nonproducer Resistance	61.3	69.3	71.3	71.3	64	65.3	65.3	65.3	4	61	61	74	74	71.3

Fig 5: Comparative study of antibiotic susceptibility pattern in both MBL producer and MBL non producer
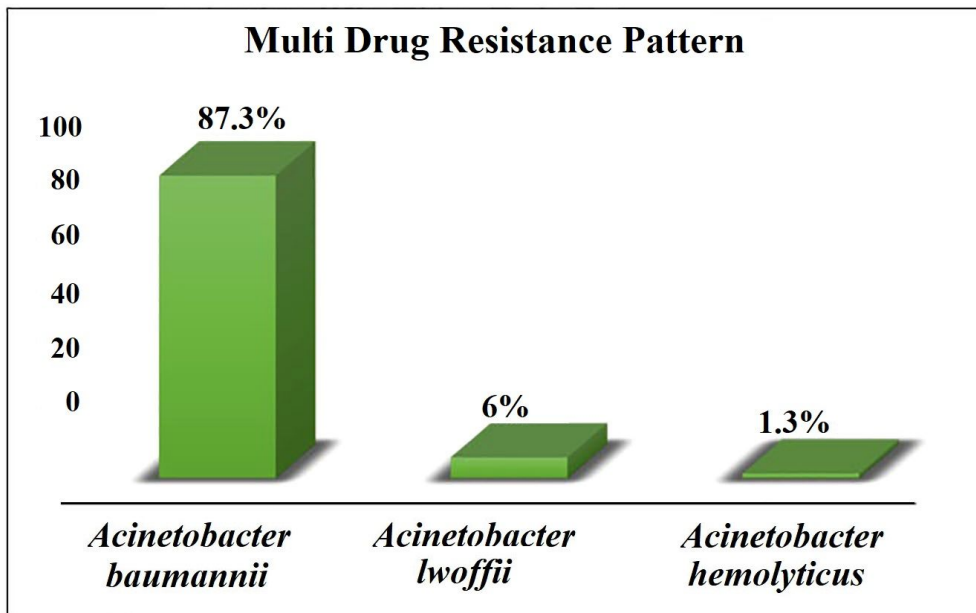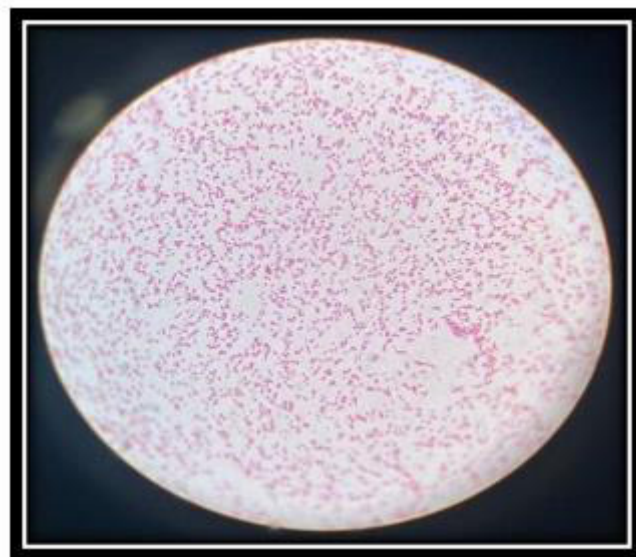
Fig 6: Multi-Drug Resistance Pattern

Fig 7: Gram-negative coccobacilli

Fig 8: CLED agar

Fig 9: MacConkey agar

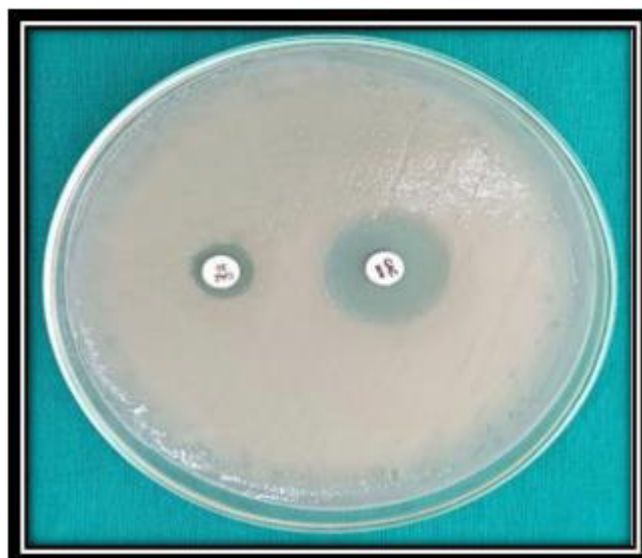**Fig10: Biochemical Tests from left to right – TSI, Indole, MR, Nitrate reduction, Citrate, Urease, VP.**

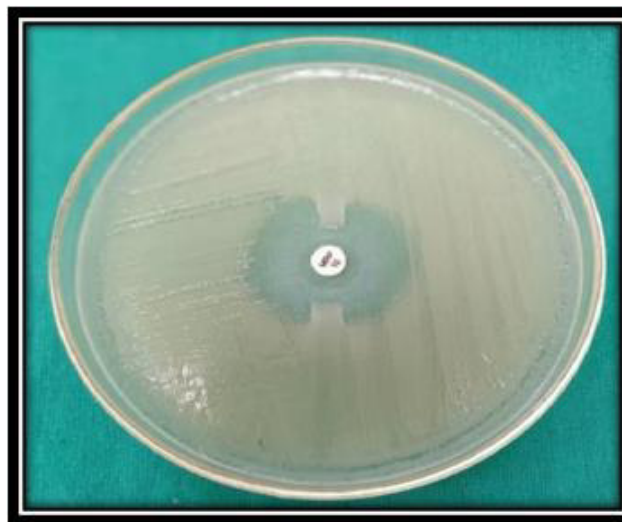**Fig 11: ESBL production**

Fig 12: MBL production

Fig 13: Modified Hodge Test

4. DISCUSSION

A total of 450 samples were collected during the study period. Out of the 450 samples,150 samples were positive for *Acinetobacter* species. A total of 150 isolates of *Acinetobacter* species were processed. There were 150 patients included in the study, of which, males were 100(67%) and 50(33%) females. A maximum number of male cases, 42(28%), were in the age group 41-60. Maximum isolates were from females aged 21-40, 18(12%). 42(28%) *Acinetobacter* species isolates were from the 41-60 age group with male predominance. In our study, a total of 150 isolates, in the majority of infections, were in the age group of 41-60 years (38%). Similar results were reported by Swarnatrisha Saha et al. [21] showing a majority of isolates in the age group 41-60 years (35%). In our study, most of the *Acinetobacter* isolates obtained were from pus sample 38(25%), followed by tracheal aspirates 36(24%), urine 30(20%), sputum 16(11%), blood 16(11%), body fluids 12(8%), CSF 2(1%). This finding can be correlated with the study conducted by B Apoorva et al. [22] in which they reported a maximum number of isolates from pus 22.5%, followed by blood 17.5%, endotracheal aspirate 17.5%, urine 15%, sputum 12.5%, BAL (Bronchoalveolar lavage) 5%, swab (gluteal abscess) 2.5%, throat swab 2.5%, CVP tip 5%. Similarly, Sana Islahi et.al[23]

reported that most isolates were from pus samples 36.95%. Shivaranjani V et al. [24] also reported maximum isolation from pus sample 38.5%. *Acinetobacter* was most commonly isolated from ICU 52(35%) mainly Surgical 41(27.3%), Neurology 24(16%), OBGY 6(4%), Pediatric 6(4%), Orthopedic 6(4%), Oncology 4(3%), Radiology 3(2%), Casualty 3(2%), Medicine 2(1%), Cath lab 2(1%), CVTS 1(0.7%). *Acinetobacter* subspecies *Acinetobacter baumannii* 138(92%) was the most common species isolated, followed by *Acinetobacter lwoffii* 10(7%) and *Acinetobacter hemolytic* 2(1.3%). The gender wise observation showed *Acinetobacter baumannii* in males 91(61%), females 45(30%), followed by *Acinetobacter lwoffii* in males 5(3.3%), females 5(3.3%) and *Acinetobacter hemolyticus* in males were 1(0.7), females 1(0.7%). The above results elucidated that maximum resistance was observed to Ampicillin 130 (87%), Nalidixic acid 128 (85.3%), Ceftazidime 126 (84%), and Cefepime 124 (83%). Maximum sensitivity was seen to Colistin 113 (75.3%) and Tigecycline 106 (71%). In the present study, *Acinetobacter species* were found to be resistant to the most commonly used antibiotics. In our study, *Acinetobacter* showed an extremely high degree of resistance to Ampicillin (87%), Nalidixic acid (85.3%), Ceftazidime (84%), Cefepime (83%), Ciprofloxacin (79%), Piperacillin (79%), Gentamicin (75%), Amikacin (73.3%), Meropenem (75%), Imipenem (71%) which correlates with

the studies by Guckan R et al. [25]. Peymani et.al[26] found that the resistance to Piperacillin +Tazobactam was 89%, Ticarcillin +Clavulanic acid 83%, Ceftazidime 92%, Cefepime 88%, Ceftriaxone 94%, Meropenem 56%, Imipenem 54%, Gentamicin 86%, Amikacin 81% and Ciprofloxacin 86%. These findings were similar to the results of our study. It shows that the extensive use of carbapenems has created a selective antibiotics pressure, increasing the prevalence of carbapenems-resistant *Acinetobacter* (CRA). The findings of our study showed 15% ESBL production, comparable to the study by Owlia P et al. [27] documented 21% ESBL production, and MBL production was 53%, similar to the findings by Amrita Talukdar et al. 9, documenting 53% MBL production. Most of the *Acinetobacter* species were Multi-Drug Resistant and also Carbapenem group resistant. The *Acinetobacter* are the ones showing the highest resistance to Carbapenems. The Modified Hodge test observed by K Lee et al. [28] was 73%, Amjad A et.al[29] 169%, A.V Kumar et al. [30] 71%, Muneeza Anwar et al. [31] 83.3%, Mojtaba Moosavian et al. [32] 53%, J. Thiriveni et al. [33] 45% and 14.8% in S John et al.[34] The present study revealed a 7% positive result for MHT. Compared to the further studies, the present study exhibited less positive percentage for MHT. In the present study, out of 150 *Acinetobacter* species isolated, 95% were found to be Multi-drug resistant (MDR). In a study conducted by Mostofi S.et.al[35], 54% of isolates were multidrug-resistant (MDR). Other studies conducted by Rekha S et al. [36] noted 74% MDR, A. S. Mathai et al. [37] observed 70% MDR, and Dash M et al. [38] studied 55% MDR. Bhattacharyya S et al. [39] and I. D. Khan et al. [40] reported 29% and 88.20% MDR isolates, respectively. Thus, Drug formulation needs to be changed according to the MDR pattern to treat more serious problems like ventilator-associated pneumonia, a nosocomial infection among critically ill patients. *Acinetobacter baumannii* is one of the most prevalent VAP-causing pathogens. Ventilator-associated pneumonia is one of the most common ICU-acquired infections, associated with a prolonged duration of microbial treatment, length of hospital stays (LOS), mechanical ventilation (MV), and a high mortality rate.

5. CONCLUSION

The study showed that ESBL production in *Acinetobacter* was 22(15%) and MBL 80(53%) and is on the rise across the globe, thus making these infections difficult to treat. The detection of ESBL and MBL production would be important for the reduction of mortality rate and spread of multidrug-resistant organisms. In the present study, *Acinetobacter*

species isolates showed higher resistance to carbapenems such as Imipenem and meropenem. Most *Acinetobacter* isolates were found to be Multi-Drug Resistant (MDR), i.e., resistant to more than or equal to three antibiotics. MDR *Acinetobacter* is widely increasing due to the inappropriate use of antibiotics in healthcare hospitals. MDR *Acinetobacter* isolates were susceptible to Colistin and Tigecycline, which can be used as a treatment for patients. The modified Hodge test is an easy and simple test that can be performed to detect carbapenemase-producing bacteria. Active surveillance combined with education of the health care worker on hand hygiene, environmental cleaning, contact precautions, antimicrobial stewardship, and handwashing practice will help curtail this organism in hospital settings.

6. ETHICAL APPROVAL STATEMENT

Ethical and research clearance was procured from the Institutional Ethics Committee, Krishna Institute of Medical Sciences, Deemed University Karad, for conducting the present research, with protocol number (054/2021-22). All the participants were informed about the research and the study, conducted to provide the required data and informed consent before participating.

7. DATA AVAILABILITY

The article contains the appropriate and proper data obtained during the experiment, supporting the research article's result, discussion, and conclusion.

8. AUTHOR'S CONTRIBUTION STATEMENT

Miss Manasi Vikas Yadav: Conceptualization, Designing the study Methodology. Dr. Geeta Satish Karande: Supervision, Writing-Reviewing, Software, Data curation, Editing, and provided valuable input towards designing the manuscript. Dr. S.R. Patil: Writing-Reviewing, Data curation, and Editing. Dr. R.V. Shinde, Dr. S.K. Pawar, Dr. H.V. Patil, Dr. P. M. Mane: Editing, Visualization, investigation, Conceptualization. Shanu Sharma: Writing- Original draft preparation, Editing, Data curation, submission, and looking for the manuscript's progress.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

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