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RESEARCH ARTICLE OPEN ACCESS

Prevalence of Extended-spectrum Beta-lactamase (ESBL), Metallo Beta-lactamase (MBL) and Carbapenemase Producing *Acinetobacter* Species Isolated from Various Clinical Samples in Tertiary Care Hospital

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Abstract

***Acinetobacter* is an important nosocomial pathogen causing health care associated infections. It is highly antibiotic resistant gram-negative bacilli. The study was done to determine the prevalence of *Acinetobacter* species isolated from various clinical samples with their antibiotic susceptibility pattern. To determine the antimicrobial susceptibility pattern of the isolated *Acinetobacter* species and also the multidrug resistant mechanisms by phenotypic characterization. The retrospective study was carried out in patients diagnosed with *Acinetobacter* infection in the Microbiology Department, Krishna Institute of Medical Sciences, Krishna Vishwa Vidyapeeth, Deemed To Be University, Karad, a tertiary care hospital, including the clinical departments, during the period of two years from November 2020 till November 2022. Organism identification, biochemical test, antibiotic resistance pattern and phenotypic tests such as ESBL, MBL and Carbapenemase production were performed as per the Clinical and Laboratory Standards Institute (CLSI). The Modified Hodge test (MHT) was performed for Carbapenemase production detection in *Acinetobacter* species. A total of 150 *Acinetobacter* species were isolated from clinical samples. *Acinetobacter baumannii* was the most prevalent species 138 (92%), followed by *Acinetobacter lwoffii* 10 (7%) and *Acinetobacter hemolyticus* 2 (1%). The isolates showed highest resistance to Ampicillin 130 (87%) and sensitive to Colistin 113 (75.3%). Most of the isolates of of *Acinetobacter baumannii* showed maximum ESBL 21(14%) and MBL 75 (93%) production. Modified Hodge test showed positive results in *Acinetobacter baumannii,* only 11 (7%). The study showed that *Acinetobacter baumannii* was the most prevalent species showing drug resistance by phenotypic detection methods. At present *Acinetobacter* is a frequent pathogen in hospital acquired infections in critically ill patients admitted to ICU. The isolates of *Acinetobacter* species in our study showed resistant to most commonly used antibiotics. The study showed that ESBL production in *Acinetobacter* was 22 (15%) and MBL 80 (53%). Most *Acinetobacter* isolates were Multi Drug Resistant (MDR). MDR *Acinetobacter* is widely increasing due to inappropriate use of antibiotics in healthcare hospital. In our study, detection of carbapenemase by Modified Hodge test was positive in 11 (7%) isolates.**

Keywords: Acinetobacter, CLSI, ICU, ATCC, ESBL, MBL, MHT, MDR

INTRODUCTION

Acinetobacter are ubiquitous, free livings saprophytes, small aerobic Gram negative cocco-bacilli that prefer moist environment and can be easily found in soil, water, food and sewage.[1] They are also ubiquitous organisms in the hospital environment, where they play a significant role in the colonization and infection of patients admitted in hospitals.[1] *Acinetobacter* causes wide spectrum of infections, including nosocomial pneumonia, secondary meningitis, surgical wound infections, skin and soft tissue infections, urinary tract infections (UTI) and bacteraemia.[2] *Acinetobacter spp.* are generally considered a part of the normal flora of the skin,[3] mucous membrane or the pharynx, and human respiratory secretions[4] and are accountable for a wide variety of local and systemic infections, including pneumonia, septicaemia and wound infections.[5] The main body areas populated by these microorganisms in hospitalized patients are the skin, oropharynx, and digestive tract. *Acinetobacter* spp. were isolated from various locations of the healthy individuals' body including the forehead, nose, ear, throat, trachea, conjunctiva, hand, vagina and perineum, inhabiting humid areas, such as axillae, the groin and toe webs.[6] *Acinetobacter* spp. are considered to be relatively low-grade bacteria.[7] In nature, the bacteria belonging to the genus *Acinetobacter,* an environmental bacterium, is widely distributed and able to survive on environmental surfaces and linked to numerous nosocomial and opportunistic infections.

The sites in which *Acinetobacter* nosocomial infections predominantly occur are contingent on the duration and local epidemiological factors present. In the preliminary reports, it has been observed that urinary tract infections (UTIs) are prevalent in intensive care units. However, it is worth nothing that the incidence of UTIs has witnessed a decline, owing

to the improved care of urinary catheters. On the contrary, there has been a considerable increase in the occurrence of nosocomial pneumonia.[8]

Of the *Acinetobacter* spp*, A. baumannii* is an important pathogen with a high morbidity and mortality, especially in the critically ill patients.[9] *Acinetobacter* spp. is associated with a wide variety of infections - ventilator-associated pneumonia (VAP), blood stream infections (BIs), urinary tract infections, bacteraemia, meningitis, skin and wound diseases, ventriculitis, cholangitis, peritonitis, and infective endocarditis. Colonization of the skin and respiratory tract by the bacteria may occur without causing an infection. The survival of *A. baumannii* in harsh environmental conditions and its ability to develop multidrug resistance attributes to making infections caused this organism highly lethal, mainly in patients who have undergone major surgeries, the immunocompromised, those with malignancy, prolonged illness and in the extremes of age.[9] Antimicrobials are chemical compounds either bactericidal or bacteriostatic, used in medical interventions to actively kill or inhibit the pathogens.

The emergence of antimicrobial-resistant *Acinetobacter* species is due both to the selective pressure exerted by the use of broad-spectrum antimicrobials and transmission of strains among patients, although the relative contributions of these mechanisms are not yet known.[10]

Infections caused by antibiotic-susceptible *Acinetobacter* isolates have usually been treated with broad-spectrum cephalosporins, β-lactam–β-lactamase inhibitor combinations (e.g., a combination that includes sulbactam, a drug marketed only in combination, in the United States), or carbapenems (e.g., imipenem or meropenem), used alone or in combination with an aminoglycoside.[11] The primary goal for the control of *Acinetobacter* infection is recognizing its presence in a hospital or long-term care facility at an early stage, controlling its spread aggressively and preventing the establishment of endemic strains. Control measures are based almost entirely on experiences from outbreaks of *Acinetobacter* infection and generally address the organism's major epidemic modes of transmission and the excessive use of broad-spectrum antibiotics.

METHODOLOGY

"Prevalence of Extended-spectrum beta-lactamase (ESBL), Metallo beta-lactamase (MBL) and Carbapenemase producing *Acinetobacter* species isolated from various clinical sample in tertiary care hospital'' study was carried out in the Microbiology department at Krishna Institute of Medical Sciences and Krishna Hospital and Medical Research Centre, Karad over a 2 years period from November 2020 to November 2022.

Inclusion criteria

Isolates of *Acinetobacter* species, from clinical samples of patients admitted with clinical infection to the IPD/OPD. Patients with both sexes involved.

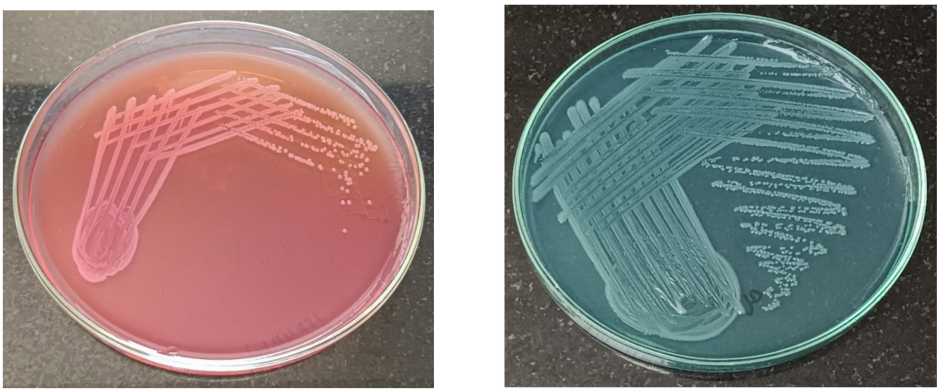


Figure 1.(a) MacConkey agar; (b) CLED agar

Exclusion criteria

To avoid duplication, isolates from the same patients and specimens were excluded.

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.

Sample collection

The various clinical samples from which *Acinetobacter* species were isolated includes -Pus, Sputum, Urine, Blood, ETT secretions, Body fluids, Wound swab, CSF and others, from all age groups and both gender of patients. Appropriate sterile containers were used for collection of the samples and then transported to the laboratory.

Bacterial identification

The clinical samples were cultured on appropriate culture media and the organisms isolated were identified using standard Microbiology procedures.[12] Nutrient agar, MacConkey agar, (Figure 1a and 1b), Blood agar, Chocolate agar were used for inoculation of the clinical samples and incubated at 37°C for 24 hours. The isolates were then identified based on colony morphology on agar and Gram stain of the smear of colonies (Figure 2). Oxidase, Catalase reactions were performed (Figure 3). Further Biochemical reactions carried out for identification of the organisms (Figure 4a, 4b and Figure 5).[12]

Antibiotic susceptibility testing

Antimicrobial susceptibility testing by Disc diffusion method of Kirby-Bauer's was carried out on Muller Hinton agar. The opacity adjusted to 0.5 McFarland standard. The antibiotic discs used were Amikacin (30 µg), Ciprofloxacin (5 µg), Cefepime (30 µg), Piperacillin (100 µg), Imipenem (10 µg), Meropenem (10 µg), Gentamicin (5 µg), Levofloxacin (5 µg), Tigecycline (15 µg), Colistin (10 µg), Co-trimoxazole (25 µg), Nalidixic acid (30 µg), Ampicillin (10 µg), Ceftazidime (30 µg) (Figure 6a and 6b). CLSI guidelines were used for the interpretation of the Zone diameter.[13,14]

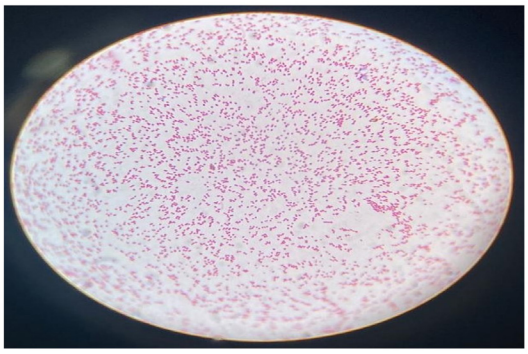


Figure 2. Gram negative coccobacilli

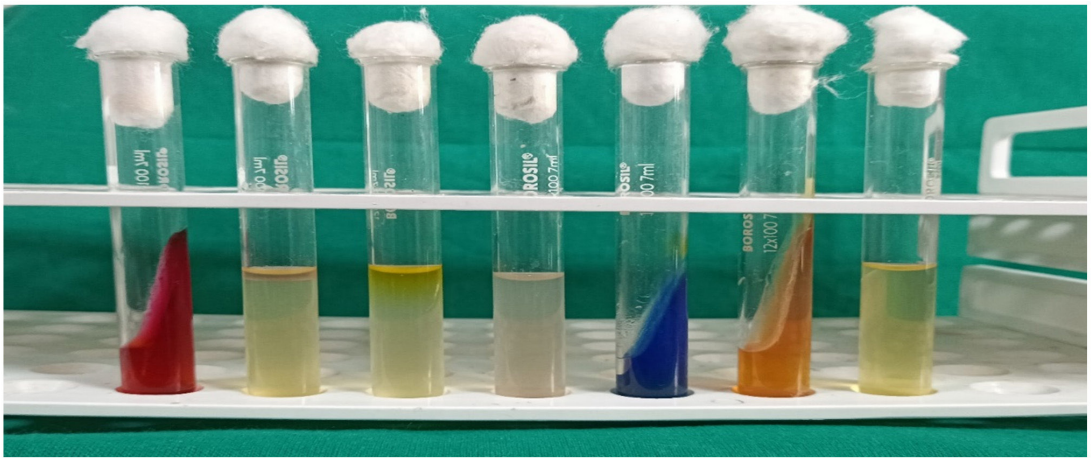
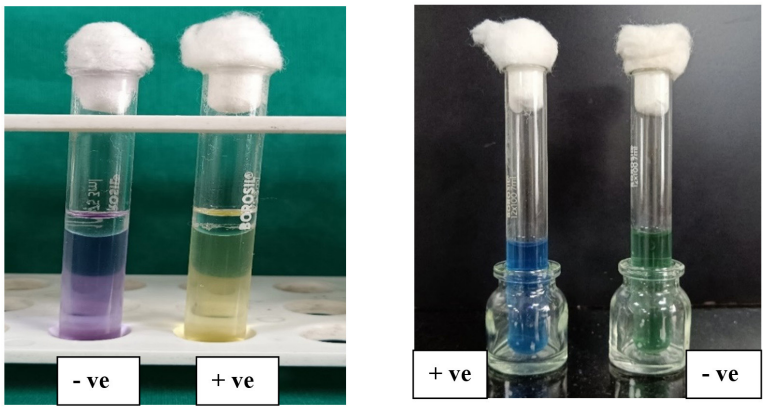


Figure 3. Biochemical tests from left to right
TSI, Indole, MR, Nitrate reduction, Citrate, Urease, VP

Extended Spectrum of Beta Lactamase (ESBL) production testing

Double disc synergy test

Double disc synergy test (DDST) was performed for testing ESBL production, using Ceftazidime + Clavulanic acid along with Ceftazidime (Cephalosporin). Test and control organism inoculum was prepared and matched with 0.5 McFarland turbidity standard. The bacterial strains cultured on Mueller Hinton agar plates, as per CLSI guidelines. A disc containing Ceftazidime + Clavulanic acid (30 µg +10 µg), applied on the plate at a distance of 25mm from that of Ceftazidime (30 µg) and incubated for 18-24 hours. An increase ≥ 5 mm in the inhibition diameter of the ceftazidime disc applied after pre-diffusion of Ceftazidime-Clavulanic acid in comparison with ceftazidime disc, considered as positive for ESBL production (Figure 7a).[15]



(a) (b)

Figure 4. (a) Arginine hydrolysis test; (b) Malonate utilization test

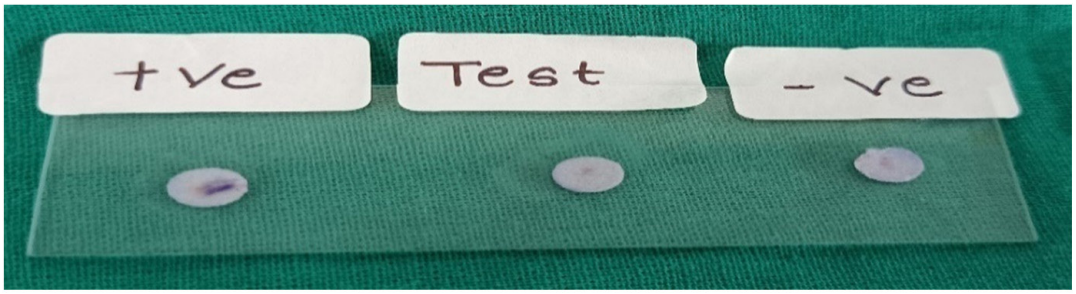
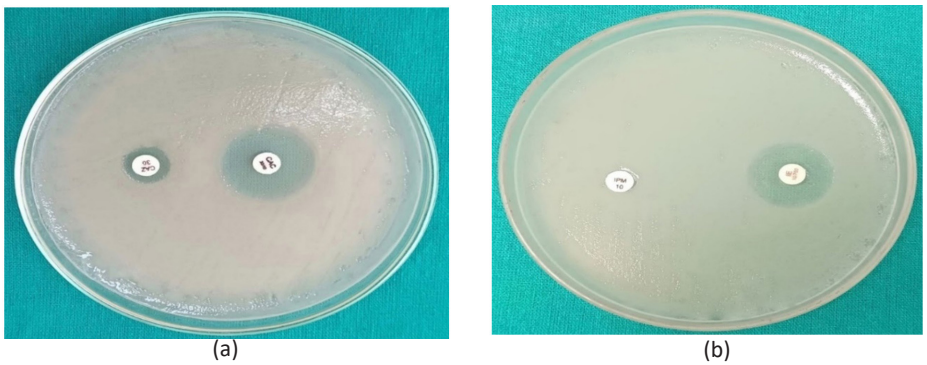


Figure 5. Oxidase Test



(a) (b)

Figure 6. Antibiotic Sensitivity Test

Positive control

Klebsiella pneumoniae ATCC 700603.

Negative control

Escherichia coli ATCC 25922.

Metallo-β-lactamase detection (MBL) testing

Imipenem-EDTA combined disc diffusion test:

The screening test for the detection and confirmation was tested by Imipenem-EDTA combined disc diffusion test. Test and control organism inoculum was prepared and matched with 0.5 McFarland turbidity standard. The bacterial strains cultured on Mueller Hinton agar plates, as per CLSI guidelines. Imipenem 10 µg disc, placed 20 mm apart, center from an Imipenem + EDTA disc containing 0.5 µl of 0.5 mg EDTA (750 µg per disk). Plates incubated at 37°C for 16-18 hours. On overnight incubation, an increase in zone size ≥ 7 mm around the Imipenem-EDTA disc compared to the Imipenem disc alone was recorded positive for MBL (Figure 7b).[16]

Control strains

Pseudomonas aeruginosa ATCC 27853.

Carbapenemase production detection testing Modified Hodge Test (MHT)

Meropenem 10 µg disc placed in the center of the Muller Hinton agar plate and 10 µl of 50 mg Zinc sulfate solution added to the Meropenem disc and incubated at 37°C overnight. Zone around Meropenem disc with clover leaf-like indentation, was interpreted as positive for Carbapenemase detection by the Modified Hodge Test (Figure 7c).[17]

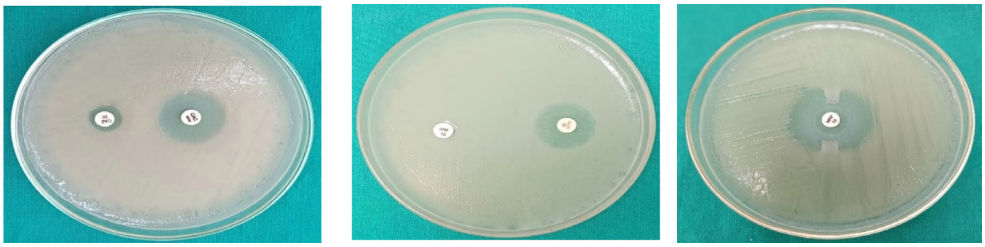


Figure 7. (a) ESBL production (DDST); (b) MBL production (CDDT); (c): Carbapenemase production (MHT)

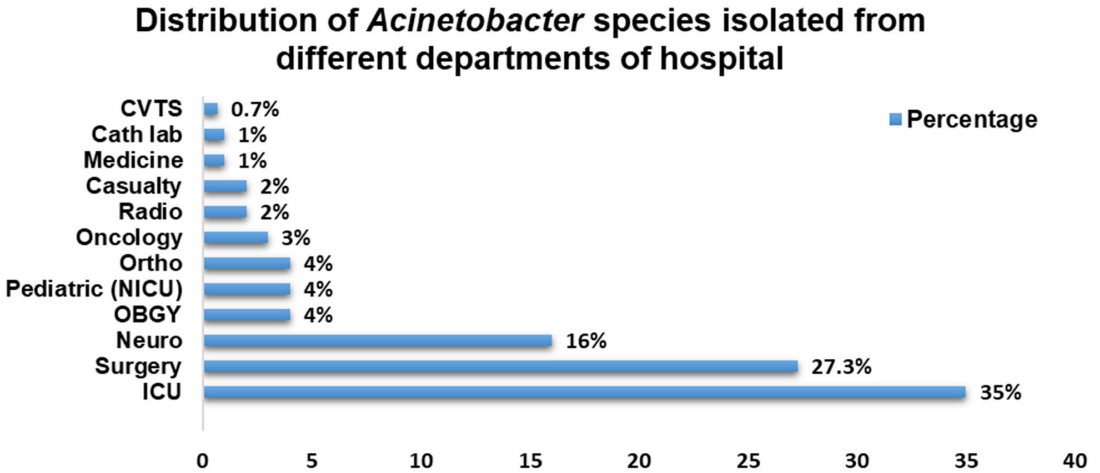


Figure 8. Distribution of *Acinetobacter* species isolated from different departments of hospital

Maximum isolates were from ICU 52(35%), followed by Surgery 41 (27.3%), Neuro 24 (16%), OBGY 6 (4%), Pediatric (NICU) 6 (4%), Ortho 6 (4%), Oncology 4 (3%), Radio 3 (2%), Casualty 3 (2%), Medicine 2 (1%), Cath lab 2 (1%), CVTS 1 (0.7%)

Meropenem 10 µg disc placed in the center of Muller Hinton agar plate and 10 µl of 50 mg Zinc sulfate solution added to the Meropenem disc and the plates incubated at 37°C overnight. Zone around the Meropenem disc with clover leaf-like indentation, was interpreted as positive for Carbapenemase production by the Modified Hodge Test.[17]

Positive control

Klebsiella pneumoniae ATCC 1705.

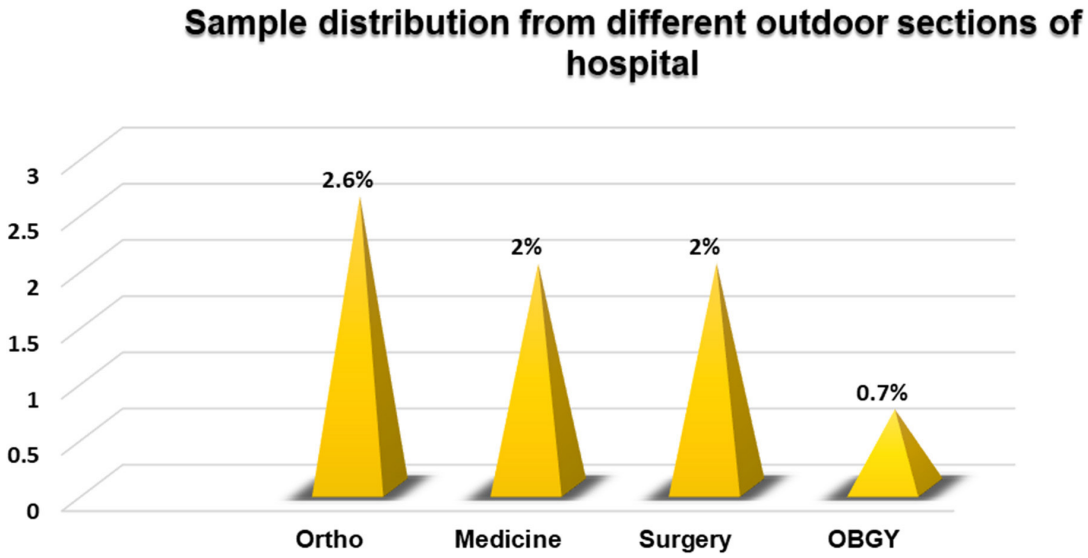


Figure 9. Sample distribution from different outdoor sections of hospital
Sample distribution from different outdoor sections of hospital. Maximum isolates were from Ortho 4 (2.6%), followed by Medicine 3 (2%), Surgery 3 (2%), OBGY 1 (0.7%).

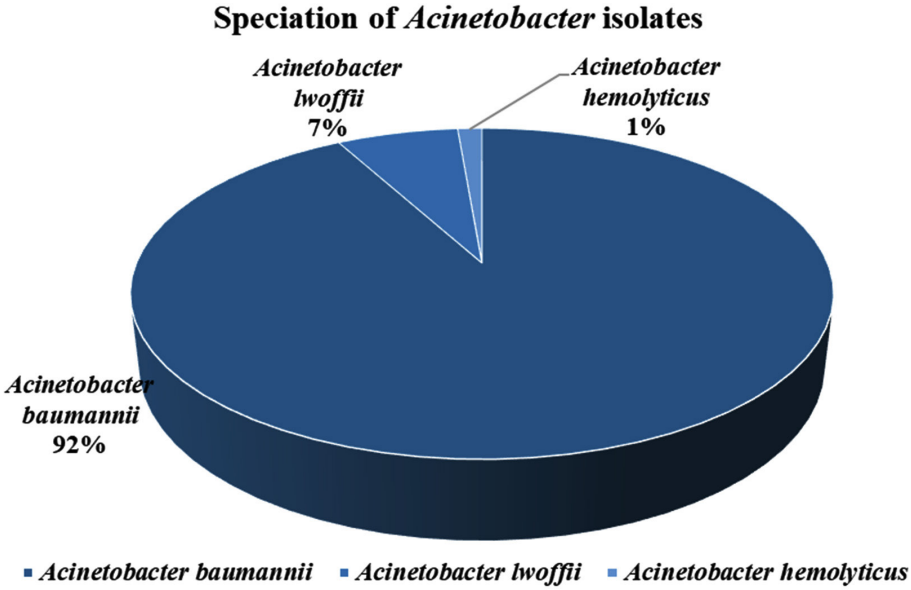


Figure 10. Speciation of *Acinetobacter* isolates
The most common species isolated was *Acinetobacter baumannii* 138 (92%), followed by *Acinetobacter lwoffii* 10 (7%) and *Acinetobacter hemolyticus* 2 (1%) respectively

Negative control

Klebsiella pneumoniae ATCC 17051706.

OBSERVATION AND RESULTS

Table 1 shows age and gender wise distribution of *Acinetobacter* isolated. The isolates in the age group of 0-20 years were 14(9%), followed by age group of 21-40 years 46(31%), 41-60 years 57(38%), >60 years 33(22%) respectively.

DISCUSSION

In the present study, a total of 150 *Acinetobacter* was identified from 450 non-lactose fermenting bacteria. Our study is comparable with

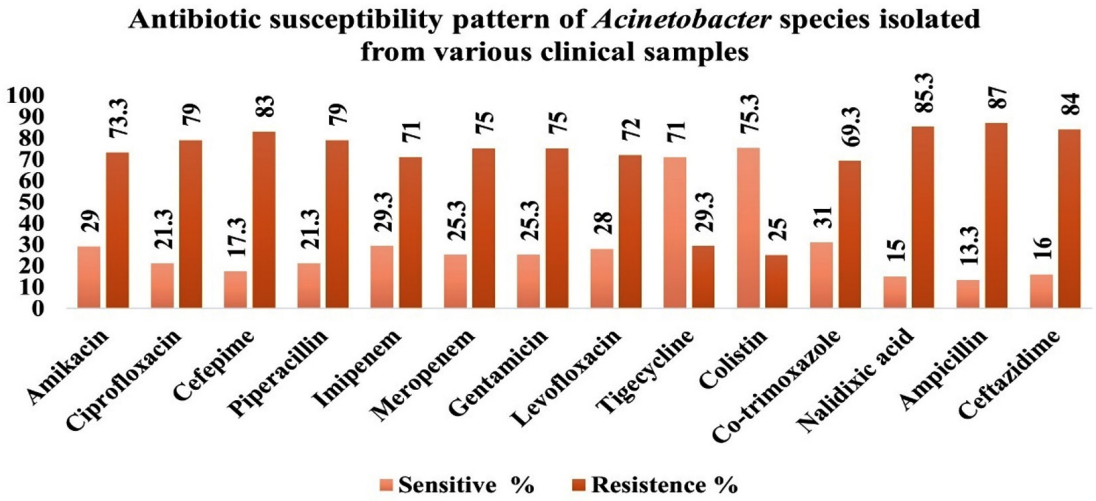


Figure 11. Antibiotic susceptibility pattern of *Acinetobacter* species isolated from various clinical samples

The different resistance pattern of bacterial isolates was observed against antimicrobial agents. Maximum sensitivity to Colistin 113(75.3%) was showed by *Acinetobacter baumannii*, followed by Tigecycline 106(71%), whereas, maximum resistance was to Ampicillin 130(87%), followed by Nalidixic acid 128(85.3%), Ceftazidime 126(84%)

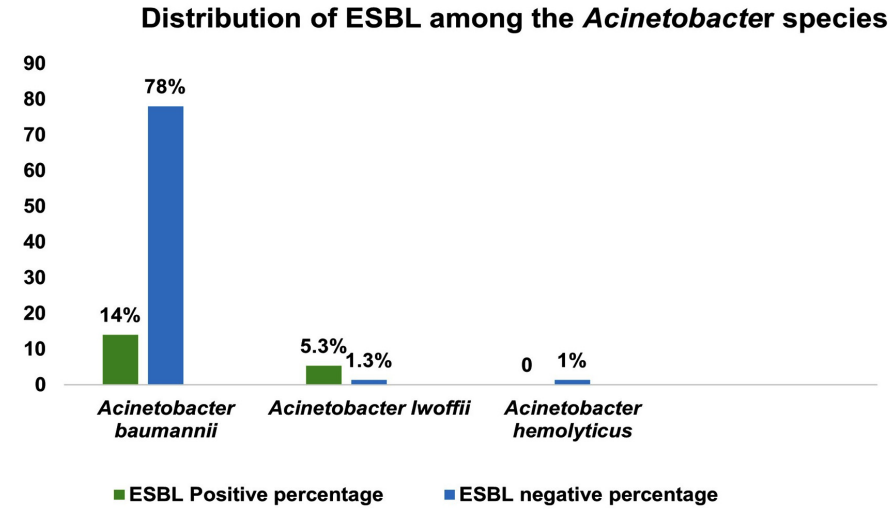


Figure 12. Distribution of ESBL among the *Acinetobacter* species

Distribution of ESBL among the *Acinetobacter* species. Maximum ESBL production was observed in *Acinetobacter baumannii* 21(14%), followed by *Acinetobacter lwoffii* 8(5.3%). Non-ESBL production were observed in *Acinetobacter baumannii* 117(78%), *Acinetobacter lwoffii* 2(1.3%) and *Acinetobacter hemolyticus* 2(1%)

other studies,[18] wherein maximum males were affected than females as given in the Table 1. The most commonly isolated species was Acinetobacter baumannii, followed by *Acinetobacter lwoffii* and *Acinetobacter hemolyticus* (Table 2). This finding can be corelated with the other study,[19] wherein they have been reported maximum number of isolates from pus, followed by blood, endotracheal

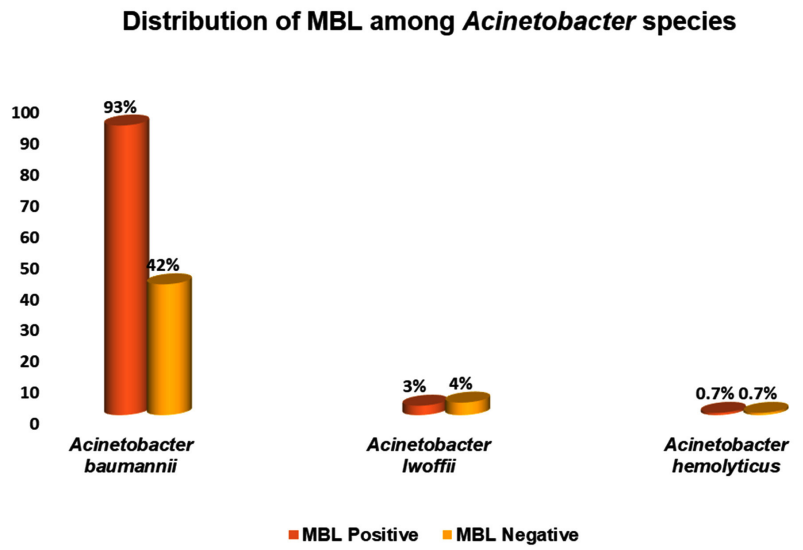


Figure 13. Distribution of MBL among the *Acinetobacter* species
Out of 150 isolates, maximum MBL positive isolates were observed in *Acinetobacter baumannii* 75(93%), followed by *Acinetobacter lwoffii* 4(3%), *Acinetobacter hemolyticus* 1(0.7%). MBL negative isolates were observed in *Acinetobacter baumannii* 64(43%), followed by *Acinetobacter lwoffii* 6(4%)

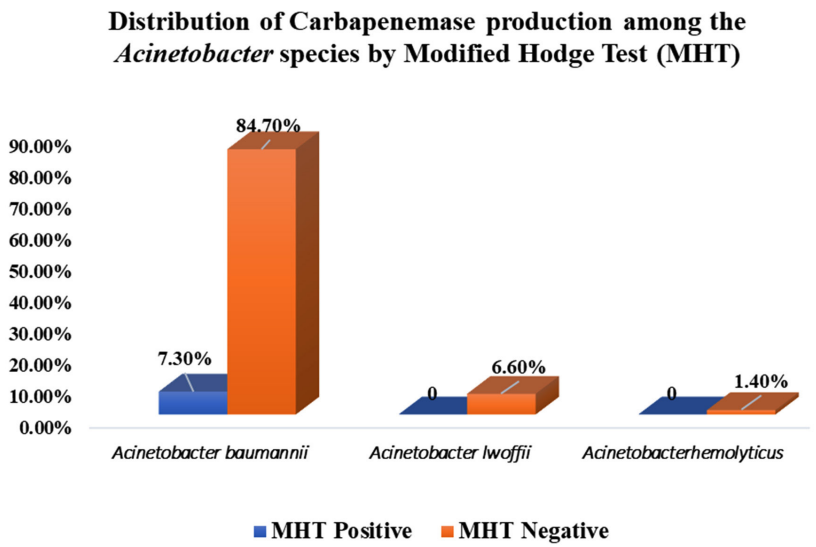


Figure 14. Distribution of Carbapenemase production among the *Acinetobacter* species by Modified Hodge Test (MHT). Modified Hodge test showed positive results in *Acinetobacter baumannii* only 11 (7%) of *Acinetobacter baumannii* were positive and 139 (93127 (84.67%)) were negative by Modified Hodge test.
Acinetobacter lwoffii and *Acinetobacter hemolyticus,* were negative for Carbapenemase production by Modified Hodge test

aspirate, urine, sputum, BAL (Bronchoalveolar lavage), swab (gluteal abscess), throat swab, CVP tip (Table 3). Similarly, other study[20] reported that majority of isolates were from pus sample (Table 4). The other study[21] has reported a similar observation of maximum isolates, from ICU (Figure 8 and 9). Other study[22] reported maximum isolates from ICU.

The most commonly isolated species was *Acinetobacter baumannii*, followed by *Acinetobacter lwoffii* and *Acinetobacter hemolyticus* (Figure 3).

Similar findings have been observed from the other study[23] reporting maximum isolate

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

Table 2. Speciation of *Acinetobacter* isolates

Species	No. of Isolates	Percentage (%)
Acinetobacter baumannii	138	92
Acinetobacter lwoffii	10	7
Acinetobacter hemolyticus	2	1
Total	150	100

Table 3. *Acinetobacter* species distribution in various IPD departments

IPD clinical samples	*Acinetobacter* species			Total
	Acinetobacter baumannii	*Acinetobacter lwoffii*	*Acinetobacter hemolyticus*	
Tracheal aspirates	38 (27.3%)	0	0	38 (27.3%)
Pus	33 (23.7%)	0	0	33 (25.3%)
Urine	26 (18.7%)	2 (1.4%)	0	28 (19%)
Sputum	12 (8.6%)	1 (0.7%)	0	13 (11%)
Body Fluids	10 (7.1%)	0	0	10 (8.7%)
Blood	09 (6.4%)	06 (4.3%)	01 (0.7%)	16 (11%)
CSF	01 (0.7%)	0	0	01(0.7%)

x^2= 46.436, p value = < 0.0001, significant.
Distribution of Acinetobacter species in various IPD departments.
Maximum isolates were from tracheal aspirates 38(27.3%), followed by pus 33(25.3%), urine 28(19%), sputum 13(11%), body fluids 10(8.7%), blood 16(11%), CSF 1(0.7%)

Table 4. *Acinetobacter* species distribution in various OPD departments

OPD clinical samples	*Acinetobacter* species			Total
	Acinetobacter baumannii	*Acinetobacter lwoffii*	*Acinetobacter hemolyticus*	
Pus	6 (54.5%)	1 (09%)	0	7 (64%)
Sputum	2 (18.1%)	0	1 (09%)	3 (27%)
Urine	1 (09%)	0	0	1 (09%)

x^2= 3.376, p value = 0.4970, Not significant.
Of the *Acinetobacter* species isolated from various OPD departments, maximum isolates were from pus 7 (25.3%) followed by sputum 3 (11%), urine 1 (19%)

from *Acinetobacter baumannii* (Figure 10). In our study, we observed that the antibiotic sensitivity pattern showed maximum sensitivity to Colistin and Tigecycline (Table 5). Similarly, a study[20] showed that most of *Acinetobacter*, were Colistin sensitive, and other study[24] reported maximum susceptibility of *Acinetobacter* to Colistin. *Acinetobacter* showed extremely high degree of resistant to Ampicillin, Nalidixic acid, Ceftazidime, Cefepime, Ciprofloxacin, Piperacillin, Gentamicin, Amikacin, Meropenem, Imipenem correlating with the other studies by Guckan and Peymani et al.[25,26] reported resistance to Ceftriaxone, Ceftazidime, Piperacillin + Tazobactam, Cefepime, Gentamicin, Ciprofloxacin, Ticarcillin + Clavulanic acid, Amikacin, Meropenem, Imipenem. It thus proves that extensive use of carbapenems has created a selective antibiotics pressure resulting in increased prevalence of carbapenems resistant *Acinetobacter* (CRA).[27]

Our study recorded that, the resistance towards Imipenem and Meropenem

Table 5. Antibiotic susceptibility pattern of *Acinetobacter* species isolated from various clinical samples

Antibiotic	Sensitive		Resistant	
	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, p value = < 0.0001, significant

Table 6. Distribution of ESBL among the *Acinetobacter* species

Species	ESBL		Total
	Positive	Negative	
A. baumannii	21 (14%)	117 (78%)	138 (92%)
A. lwoffii	8 (5.3%)	2 (1.3%)	10 (7%)
A. hemolyticus	0 (0%)	2 (1%)	2 (1%)

x^2=25.578, p value = < 0.0001, significant

Table 7. Distribution of MBL among the *Acinetobacter* species

Species	MBL		Total
	Positive	Negative	
A. baumannii	75 (93%)	63 (42%)	138 (92%)
A. lwoffii	4 (3%)	6 (4%)	10 (7%)
A. hemolyticus	1 (0.7%)	1 (0.7%)	2 (1.3%)

x^2=0.7803, p value = 0.6770, Not significant

Table 8. Distribution of Carbapenemase production among the *Acinetobacter* species by Modified Hodge Test (MHT)

Species	MHT	
	Positive	Negative
A. baumannii	11 (7.3%)	127 (84.7%)
A. lwoffii	0	10 (6.6%)
A. hemolyticus	0	2 (1.4%)

(Figure 4 and Figure 11). Other study[23] reported high carbapenem resistance; resistant to Imipenem and Meropenem. The findings of our study showed ESBL production, comparable to the other study[28] documenting same ESBL production (Table 6 and Figure 12).

In the present study, MBL production was similar to the findings by other study[29] documenting MBL production (Table 7 and Figure 13).

The present study showed Carbapenemase production in *Acinetobacter baumannii* isolates, by Modified Hodge Test (Table 8 and Figure 14). As compared to the other study,[30] present study exhibited less positive percentage for Carbapenemase detection.

CONCLUSION

At present, *Acinetobacter* is common threat in health care associated infections particularly in critically ill ICU patients. Maximum percentage of *Acinetobacter* isolates were from pus sample followed by tracheal aspirates. *Acinetobacter baumannii* was the most common bacterial isolate among the *Acinetobacter* species. Our study showed that *Acinetobacter* species were resistant to most of the commonly used antibiotics such as Ampicillin, Nalidixic acid and Ceftazidime.

It thus proves that extensive use of carbapenems has created a selective antibiotics pressure resulting in increased prevalence of carbapenems resistant *Acinetobacter* (CRA). *Acinetobacter,* reported as MDR*,* showed susceptibility to Colistin and Tigecycline, which remains the drug of choice in the treatment for patients. All isolates were sensitive to Colistin 113 (75.3%) and resistant towards Ampicillin 130 (87%). Ceftazidime has been proposed as the indicator of ESBL production as compared to other antibiotics. The remaining isolates showed resistance to Ceftazidime.

Thus, Ceftazidime is a superior indicator for the detection of ESBL production. Imipenem has been suggested as the indicator of MBL production in comparison to others. So, Imipenem is a better indicator for the detection of MBL production. The study showed that ESBL production in *Acinetobacter* is 22(15%) and MBL 80(53%) and is on the ascent world over, in this way making these infections challenging to treat. ESBL and MBL production detection would be important for decreasing death rate and spread of multidrug resistant organisms. *Acinetobacter* species isolates were resistant to carbapenems such as imipenem 106(71%) and meropenem 112(75%). Modified Hodge test is a simple and easy test to be performed to identify carbapenems producing organisms. In our study, detection of Modified Hodge test by carbapenemase was positive in 11 (7%) isolates.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

The study was approved by the Institutional Ethical and Research Committee, Krishna Institute of Medical Sciences, Deemed University Karad, for conducting the present research, with protocol number (054/2021-22).

INFORMED CONSENT

Written informed consent was obtained from the participants before enrolling in the study.

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