Int.J.Curr.Microbiol.App.Sci (2025) *14*(08): 186-197

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 14 Number 8 (2025)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2025.1408.017

Virulence Traits, Colistin Resistance and Carbapenemase Production in Multidrug-Resistant *Acinetobacter baumannii:* A Phenotypic Study from South India

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Keywords

Multi drug resistance, Acinetobacter, Carbapenemase, Colistin, Virulence, Biofilm, Antimicrobial resistance

Article Info

Received:
08 June 2025
Accepted:
24 July 2025
Available Online:
10 August 2025

A B S T R A C T

Multidrug resistant (MDR) *Acinetobacter* is an important pathogen which has a number of virulence characteristics that enable them to render resistance to antibiotics thus complicates the treatment options, thereby forcing clinicians to rely on last resort antibiotic such as colistin. However, resistance to colistin has also been reported, further increasing the challenge. To detect the virulence attributes, colistin resistance and carbapenemase production in MDR *Acinetobacter baumannii* from a tertiary care hospital in South India. This is a prospective study conducted over a period of 15 months from August 2023 to October 2024 with 110 isolates of MDR *Acinetobacter baumannii*. All the isolates were subjected to colistin resistance detection using microbroth dilution method, carbapenemase detection using mCIM and eCIM methods, virulence detection like biofilm production by tissue culture plate method, haemolytic activity on blood agar, proteolytic activity on milk agar and siderophore production on Chrome Azurol S agar. Of the 110 isolates of MDR *A. baumannii*, 19 (17.27%) showed colistin resistance, 89 (80.90%) showed biofilm production, 35 (31.81%) showed haemolysis, 60 (54.54%) showed proteolysis and 49 (44.54%) showed siderophore production. The serine carbapenemase from mCIM and metallo beta-lactamase producers from eCIM tests were 61 (55.45%) serine carbapenemase and 70 (63.63%) respectively. This study underscores the concerning prevalence of virulence and resistance determinants among MDR *A. baumannii*, providing clinicians and infection control specialists with essential data to guide the formulation of effective empirical treatment strategies and infection control measures.

Introduction

Acinetobacter is a Gram-negative, non-motile, aerobic coccobacillus causing bloodstream, skin, soft tissue, wound infections, and ventilator-associated pneumonia (VAP), particularly in ICU patients (Lee *et al.,* 2017). Multidrug-resistant *A. baumannii* (MDRAB) has acquired resistance to most antibiotics, including

carbapenems, the preferred treatment for severe infections (Nemec *et al.,* 2015; Vijayakumar *et al.,* 2019). However, rising carbapenem-resistant *A. baumannii* (CRAB) rates have reduced treatment efficacy and increased mortality. Resistance is often mediated by oxacillinases (OXA-23-like, OXA-24-like, OXA-58-like) and the intrinsic *blaOXA-51-like* gene, which requires insertion sequences for carbapenem resistance. Other mechanisms include carbapenem-hydrolyzing β-lactamases and extended-spectrum β-lactamases (ESBLs) (Holt *et al.,* 2016; Poirel *et al.,* 2017).

Phenotypic assays such as the modified carbapenem inactivation method (mCIM) and EDTA-modified carbapenem inactivation method (eCIM), recommended by CLSI, differentiate serine β-lactamases from metallo-β-lactamases, aiding in targeted therapy and infection control (Holt *et al.,* 2016).

The global spread of CRAB has renewed reliance on polymyxins, especially colistin, as a "last-resort" antibiotic. However, colistin resistance is increasing (Poirel *et al.,* 2017). Colistin acts by binding the negatively charged lipid A in lipopolysaccharides via electrostatic interactions.

Resistance can result from mutations in *pmrCAB* (encoding the PmrA response regulator, PmrB kinase sensor, and PmrC LPS-modifying protein) and *lpxA, lpxC, lpxD* (lipid A biosynthesis), or via plasmid-mediated *mcr* genes (*mcr-1* to *mcr-5*) encoding phosphoethanolamine transferases. These genetic changes alter LPS structure, reducing colistin binding and rendering treatment ineffective (Fleming *et al.,* 2017; Rumbo-Feal *et al.,* 2013).

A. baumannii is notable for its ability to survive on inanimate surfaces thereby contributing virulence attributes like outer membrane proteins (OMPs), biofilm formation, siderophore production, haemolytic and proteolytic activity etc, which enhance its ability to cause severe infections (Holt *et al.,* 2016).

Biofilm in *Acinetobacter baumannii* is formed by a surface-attached microbial community encased in an extracellular matrix and regulated via complex gene networks like *bap*, *ompA*, *epsA*, *csuE*, *bfmS*, and genes responsible for pilus formation. Biofilms enhance colonization and resistance to antimicrobial agents, complicating treatment (Thummeepak *et al.,* 2016; Amin *et al.,* 2019). Haemolytic activity is mainly mediated by *plc1* and *plc2*, which encode phospholipase C enzymes that lyse red blood cells (Harding *et al.,* 2018; Smith *et al.,* 2007). The *ctp* gene contributes to proteolytic activity, membrane integrity, stress adaptation and virulence regulation (Antunes *et al.,* 2011). Siderophore-mediated iron uptake via acinetobactin synthesis genes (*bas*, *bar*, *bau*) also plays a vital role in pathogenicity (Peleg *et al.,* 2008; Eze *et al.,* 2018).

Therefore this study aims to assess the phenotypic characterization of virulence factors, colistin resistance and carbapenemase production in *Acinetobacter baumannii* from both clinical and environmental sources which is essential to gain insights into the organism's pathogenic potential and its ability to persist and spread within healthcare settings.

Materials and Methods

This is a prospective study conducted in a tertiary care hospital, South India. Clinical and environmental MDR *Acinetobacter baumannii* were collected for over a 15-month period, from August 2023 to October 2024. Samples such as sputum, endotracheal aspirates, pus, blood, urine, and other body fluids were processed according to established standard protocols.

Multidrug-resistant *A. baumannii* isolates were identified using the VITEK-2 system (BioMérieux, India), in accordance with the manufacturer's guidelines (BioMérieux India, 2025).

The study included 55 clinical MDR *A. baumannii* isolates along with 55 corresponding environmental isolates were isolated.

Patient's surroundings from whom MDR *Acinetobacter* were isolated clinically, were screened for the presence of MDR *Acinetobacter spp*. From each patient 5 environmental samples (Wall, trolley, floor, bathroom, bed and bed rails) were obtained. MDR *Acinetobacter* isolated from all or any one patient environmental site were considered.

All the isolates were subjected to colistin resistance detection using microbroth dilution method. Using the MIKROLATEST kit (LOT: 1710152) and CLSI 2023 guidelines with the proper controls (ATCC Ps. aeruginosa 27853 and ATCC E. coli 25922), the broth microdilution method was carried out (MICROXPRESS, 2025).

Carbapenemase detection

mCIM (Modified Carbapenem Inactivation Method): For each isolate, 1-µL loopful of test organism from an overnight blood agar culture was emulsified in 2 mL tryptic soy broth (TSB). A 10-µg meropenem disc was added and vortexed for 10–15 s, later incubated at 35 °C ± 2 °C for 4 h 15 min. A 0.5 McFarland suspension of *E. coli* ATCC® 25922 was prepared in saline or broth, inoculated onto Mueller–Hinton agar (MHA), and allowed to dry. The disc was then transferred from the TSB to the MHA, then incubated for 18–24 h at 35 °C ± 2 °C and measured for the inhibition zone (Rajshekar *et al.,* 2024).

eCIM (EDTA-Modified Carbapenem Inactivation Method): To reach a final concentration of 5 mM, a second 2-mL TSB tube was labelled and 20 µL of 0.5 M EDTA was added. Proceeded further as per the mCIM protocol, processing the two tubes simultaneously and putting the meropenem discs from the mCIM and eCIM on the same MHA was followed (Rajshekar *et al.,* 2024).

mCIM and eCIM result interpretation

eCIM Results (to be interpreted only if mCIM is positive):

Metallo-β-lactamase positive: The zone diameter increases by ≥5 mm in comparison to the mCIM, indicating the presence of metallo-β-lactamase. The inhibition of *E. coli* ATCC® 25922 is a result of EDTA's suppression of the enzyme's activity.
Metallo-β-lactamase negative: The activity of the enzyme is not significantly affected by EDTA, and an increase of ≤4 mm in the zone diameter relative to the mCIM indicates the presence of a serine carbapenemase (Rajshekar *et al.,* 2024).

Reporting of mCIM and eCIM

- ✓ For carbapenemase-positive isolates, report as "Carbapenemase positive," including the zone diameter and any pinpoint colonies.
- ✓ For carbapenemase-negative isolates, report as "Carbapenemase negative," with the clear zone diameter.
- ✓ For inconclusive results, report as "Carbapenemase inconclusive" and recommend additional testing.
- ✓ For eCIM, if metallo-β-lactamase positive, report as "Metallo-β-lactamase positive" with the zone diameters for both mCIM and eCIM. If metallo-β-lactamase negative, report as "Metallo-β-lactamase negative," including the zone diameters for both tests (Rajshekar *et al.,* 2024).

Biofilm formation was assessed using the tissue culture plate method as per Christensen *et al.,* (1985). The test organism was adjusted to a 0.5 McFarland standard, diluted 1:100 in tryptic soy broth, and 200 µL was inoculated into 96-well plates, followed by incubation at 37 °C for 24 h. The wells were rinsed three times with PBS, air-dried, fixed using 2% sodium acetate, and stained with 0.1% crystal violet. Any excess stain was removed by washing with PBS, and the bound dye was subsequently dissolved in 30% acetic acid. Biofilm production was quantified by measuring OD at 570 nm using an ELISA reader.

Test organism, adjusted to 0.5 McFarland standard) was inoculated on 5% sheep blood agar, skim milk agar, and chrome azurol S (CAS) agar plates procured from HiMedia Laboratories, India (HiMedia Laboratories Pvt Ltd India, 2025). Incubated at 37 °C overnight, and observed for haemolysis pattern (Ko *et al.,* 2000)—indicated by a clear zone of haemolysis surrounding the bacterial colony, proteolytic activity (Jones *et al.,* 2007) was identified by the presence of yellow-colored colonies, with or without a surrounding clear zone, while siderophore production (Schwyn and Neilands, 1987)—was indicated by a color shift from blue to orange or the appearance of a yellow to light orange halo around the bacterial colony.

Results and Discussion

A total of 110 multidrug-resistant (MDR) *Acinetobacter baumannii* isolates were analyzed, comprising 55 clinical and 55 patient environmental isolates. Of the clinical isolates, 37 (67.27%) were obtained from male patients and 18 (32.72%) from female patients, resulting in a male-to-female ratio of 3:1. The highest proportion (34.54%) was obtained from patients aged 61–80 years, followed by 27.27% from 41–60 years and 30.90% from 21–40 years (Table 01). The majority (51/55; 93%) were recovered from in-patients (Table 02), of which 42 (82.35%) originated from intensive care units (ICUs). The Critical Care Medical ICU was the predominant source, contributing 12 (28.57%) isolates (Table 03). Nine isolates (17.64%) were from hospital wards, with the private ward accounting for 6 (66.66%) of these (Table 03). The primary clinical specimen was

endotracheal aspirate (35/55; 63.63%), followed by pus (9/55; 20%) and sputum (3/55; 7.27%) (Table 04). All clinical isolates were intermediate to colistin; 41 (78.18%) were sensitive to tigecycline, 10 (18.18%) to minocycline, and the majority exhibited resistance to carbapenems, fluoroquinolones, aminoglycosides, and cephalosporins (Table 05).

From 275 environmental samples collected (five per patient: wall, trolley, floor, bathroom, bed/bed rails), 55 MDR *A. baumannii*, 42 sensitive *Acinetobacter*, 37 *Escherichia coli*, 41 *Klebsiella*, 36 *Pseudomonas*, and 39 MDR *E. coli* were isolated, while 25 samples showed no bacterial growth (Table 06).

Among the MDR *A. baumannii* environmental isolates, 24 (43.63%) were recovered from beds/rails, 16 (29.09%) from floors, 8 (14.54%) from walls, 5 (9.09%) from trolleys, and 2 (3.63%) from bathrooms. All environmental isolates showed intermediate susceptibility to colistin; 26 (47.27%) were susceptible to tigecycline, and 7 (20%) to minocycline (Table 07).

Colistin susceptibility testing by the microbroth dilution method revealed that 43 (78.19%) of the clinical MDR *A. baumannii* isolates and 48 (87.28%) of the environmental isolates were sensitive, while 12 (21.81%) and 7 (12.72%) isolates, respectively, were resistant (Table 08 and FIGURE 01). Carbapenemase detection by mCIM and eCIM showed that, among the clinical isolates, 36 (65.45%) produced serine carbapenemases and 40 (72.27%) produced metallo-β-lactamases. Among environmental isolates, 25 (45.5%) were positive for serine carbapenemase production and 30 (54.5%) for metallo-β-lactamase production (Table 09 and FIGURE 02).

Virulence factor analysis revealed that, among the clinical isolates, 34 (61.81%) were strong biofilm producers, 3 (5.45%) moderate, and 18 (32.72%) non-producers. In contrast, environmental isolates exhibited 6 (10.90%) strong, 19 (34.54%) moderate, and 30 (54.54%) non-biofilm producers (FIGURE 03).

Haemolytic activity was present in 20 (36.36%) clinical and 15 (27.27%) environmental isolates (FIGURE 04). Proteolytic activity was detected in 29 (52.72%) clinical and 31 (56.36%) environmental isolates (FIGURE 05). Siderophore production was positive in 30 (54.54%) clinical and 19 (34.54%) environmental isolates (FIGURE 06) [Table 10].

In this study, the phenotypic detection of virulence markers in MDR *A. baumannii* isolates from clinical and environmental sources was assessed. Clinical isolates generally exhibited stronger virulence traits than environmental isolates.

The male-to-female ratio of MDR *A. baumannii* infections was 3:1, with 67.27% from male patients. This male predominance aligns with Huang *et al.,* (2018), who reported a ratio of 3.9:1. Age distribution showed the highest infection rate in the 61–80 year group (34.54%), followed by 41–60 years (27.27%) and 21–40 years (30.90%). Similar to Yadav *et al.,* (2020), older patients were more vulnerable due to immune suppression and chronic comorbidities.

Most clinical isolates (82.35%) originated from ICU patients, consistent with Boulesnam *et al.,* (2023) (100% ICU) and Yadav *et al.,* (2020) (49.6% ICU). Endotracheal aspirates were the predominant sample type (63.63%), similar to Yadav *et al.,* (2020) (47.2% respiratory), whereas Boulesnam *et al.,* (2023) found surgical wounds most common (73.33%), reflecting differing patient profiles.

Environmental isolates were primarily recovered from beds and bed rails (63.63%), in line with Boulesnam *et al.,* (2023) (60%).

Colistin susceptibility testing revealed that most MDR *A. baumannii* isolates remained sensitive—78.19% of clinical and 87.28% of environmental strains—aligning with Nang *et al.,* (2019) and Jeannot *et al.,* (2017) but lower than resistance levels reported by Ayoub Moubareck and Hammoudi Halat (2020). Carbapenemase screening showed high rates of metallo-β-lactamase (MBL) production (clinical: 72.27%, environmental: 54.5%) compared with serine carbapenemases (65.45% and 45.5%, respectively), similar to Wu *et al.,* (2019) and Lee *et al.,* (2011). The predominance of MBLs is clinically concerning, given their broad β-lactam resistance and lack of effective inhibitors (Bassetti *et al.,* 2014). Lower carbapenemase prevalence in environmental isolates mirrors Almasaudi *et al.,* (2018), suggesting reduced direct antibiotic pressure, yet these strains remain important environmental reservoirs. The concurrent presence of carbapenemase producers and emerging colistin resistance underscores the urgent need for antimicrobial stewardship, rigorous infection control, and continuous resistance surveillance.

Table.1 The interpretation of biofilm production was classified as follows

Optical Density	Biofilm Production
>0.68	Strong biofilm producer
0.35-0.68	Moderate biofilm producer
0.17-0.34	Weak biofilm producer
<0.17	Non biofilm producer

Note: Weak biofilm producers were also considered as nonbiofilm producer.[19]

Table.2 Age wise distribution of 55 clinical MDR *A. baumannii*

0-20 years	**3 (5.45%)**
21-40 years	17 (30.90%)
41-60 years	16 (29.09%)
61-80 years	19 (34.54%)
Total	**55**

Table.3 Department distribution of 55 clinical MDR *A. baumannii*

In-Patient	**51 (92.72%)**
Out-Patient	4 (7.27%)
Total	**55**

Table.4 ICU and Ward distribution of 51 clinical MDR *A. baumannii*

Respiratory ICU	**3 (7.14%)**
Surgery ICU	7 (16.66%)
Paediatric ICU	2 (4.76%)
Burns ICU	2 (4.76%)
Critical Care Medicine ICU	12 (28.57%)
Medicine ICU	6 (14.28%)
Neuro ICU	8 (19.04%)
Neonatal ICU	2 (4.76%)
Total	**42**
Private ward	6 (66.66%)
Male surgery ward	2 (22.22%)
General male ward	1 (11.11%)
Total	**9**
Grand Total	**51**

Table.5 Sample distribution of 55 clinical MDR *A. baumannii*

Blood	**2 (3.63%)**
Pus	11 (20%)
Endotracheal aspirate	35 (63.63%)
Sputum	4 (7.27%)
Urine	3 (5.45%)
Total	**55**

Table.6 Antimicrobial susceptibility testing of 55 clinical MDR *A. baumannii*

Drug	Sensitive No.	%	Intermediate No.	%	Resistant No.	%
Colistin	12	21.82	43	78.18	0	0.00
Tigecycline	43	78.18	11	20.00	1	1.82
Minocycline	10	18.18	6	10.91	39	70.91
Gentamycin	6	10.91	2	3.64	47	85.45
Ceferazone/sulbactam	4	7.27	0	0.00	51	92.73
Amikacin	0	0.00	1	1.82	54	98.18
Cefepime	0	0.00	3	5.45	52	94.55
Levoflaxacin	1	1.82	1	1.82	53	96.36
Cotrimoxazole	2	3.64	0	0.00	53	96.36
Piperacillin/tazobactam	0	0.00	0	0.00	55	100.00
Ceftazidime	0	0.00	0	0.00	55	100.00
Ceftriaxone	0	0.00	0	0.00	55	100.00
Imipenem	0	0.00	0	0.00	55	100.00
Meropenem	0	0.00	0	0.00	55	100.00
Ciprofloxazin	0	0.00	0	0.00	55	100.00

Table.7 Distribution of 275 Environmental samples from patient surrounding.

	MDR *A. baumannii*	Sensitive *Acinetobacter*	Sensitive *E. coli*	MDR *E. coli*	Sensitive *Pseudomonas*	Sensitive *Klebsiella*	No Growth	Total
Bed & Rail	22 (40%)	5 (9.09%)	2 (3.63%)	17 (30.90%)	5 (9.09%)	1 (1.81%)	3 (5.45%)	55
Floor	18 (32.72%)	3 (5.45%)	15 (27.27%)	2 (3.63%)	8 (14.54%)	9 (16.36%)	0	55
Wall	4 (7.27%)	10 (18.18%)	12 (21.81%)	5 (9.09%)	5 (9.09%)	11 (20%)	8 (14.54%)	55
Trolley	9 (16.36%)	11 (20%)	4 (7.27%)	0	8 (14.54%)	9 (16.36%)	14 (25.45%)	55
Bathroom	2 (3.63%)	13 (23.63%)	4 (7.27%)	15 (27.27%)	10 (18.18%)	11 (20%)	0	55
Grand Total	55	42	37	39	36	41	25	275

Table.8 Antimicrobial susceptibility testing of 55 environmental MDR *A. baumannii*

Drug	Sensitive No.	%	Intermediate No.	%	Resistant No.	%
Colistin	18	32.72	37	67.27	0	0
Tigecycline	26	47.27	22	40.00	7	12.73
Minocycline	11	20.00	0	0.00	44	80.00
Gentamycin	6	10.91	4	7.27	45	81.82
Ceferazone/sulbactam	8	14.55	4	7.27	43	78.18
Amikacin	0	0.00	8	14.55	47	85.45
Cefepime	0	0.00	9	16.36	46	83.64
Levoflaxacin	0	0.00	8	14.55	47	85.45
Cotrimoxazole	1	1.82	0	0.00	54	98.18
Piperacillin/tazobactam	0	0.00	0	0.00	55	100.00
Ceftazidime	0	0.00	0	0.00	55	100.00
Ceftriaxone	0	0.00	0	0.00	55	100.00
Imipenem	0	0.00	0	0.00	55	100.00
Meropenem	0	0.00	0	0.00	55	100.00
Ciprofloxazin	0	0.00	0	0.00	55	100.00

Table.9 Colistin susceptibility testing of 55 clinical and of 55 environmental MDR *A. baumannii*

Microbroth Dilution Method	Clinical MDR A. baumannii	Environmental MDR A. baumannii
Sensitive	78.19%	87.28%
Resisatnt	21.81%	12.72%

Table.10 Carbapenemase production in 55 clinical and 55 environmental MDR *A. baumannii*

Carbapenemase production methods	Clinical MDR *A. baumannii*	Environmental MDR *A. baumannii*
mCIM positive	65.45%	45.50%
eCIM positive	72.27%	54.50%

Table.11 Virulence distribution of 55 clinical and 55 Environmental MDR *A. baumannii*

Virulence	Biofilm producer	Biofilm non producer	Haemolytic	Non haemolytic	Proteolytic	Non proteolytic	Siderophore producer	Siderophore non producer
Clinical MDR *A. baumannii*	37 (67.26%)	18 (32.72%)	20 (36.36%)	35 (63.63%)	29 (52.72%)	26 (47.27%)	30 (54.54%)	25 (45.45%)
Environmental MDR *A. baumannii*	25 (45.44%)	30 (54.54%)	15 (27.27%)	40 (72.72%)	31 (56.36%)	24 (43.63%)	19 (34.54%)	36 (65.45%)

Figure.1 Colistin Susceptibility Testing by Microbroth Dilution

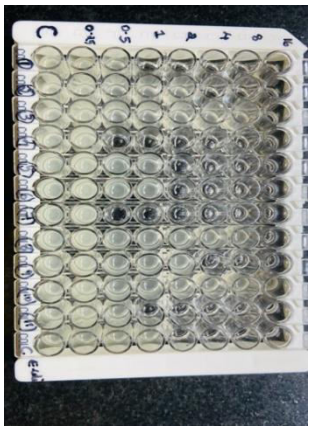


Figure.2 Carbapenemase Production by mCIM and eCIM Method.

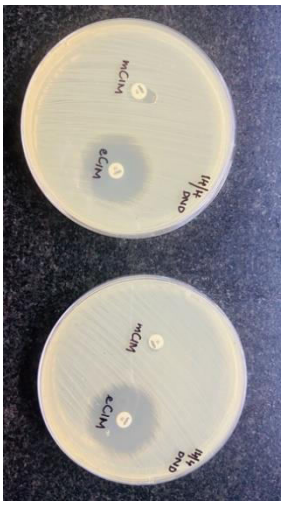


Figure.3 Biofilm Production by Tissue Culture Plate Method.

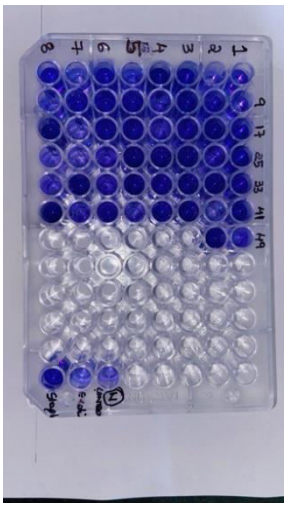
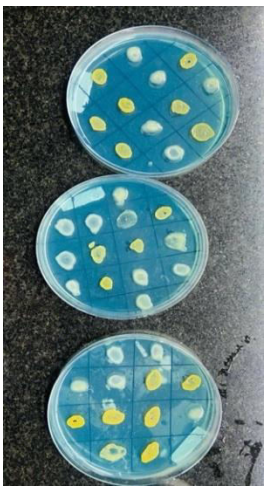


Figure.4 Haemolysis on Blood Agar.

Figure.5 Proteolysis on Milk Agar.

Figure.6 Siderophore Production on Chrome Azurol S Agar.



Biofilm production was markedly higher in clinical isolates (61.81%) than environmental ones (10.90%). In contrast, Boulesnam *et al.,* (2023) reported moderate–high biofilm potential in both environmental (56%) and clinical (50%) isolates, while Bardbari *et al.,* (2017) found 31.2% of clinical and 58.7% of environmental isolates as strong producers.

Haemolytic activity was detected in 36.36% of clinical and 27.27% of environmental isolates. Pournaras *et al.,* (2021) also observed higher haemolytic activity in clinical strains (45.5%), suggesting its importance in human pathogenicity.

Proteolytic activity was similar in both groups (clinical 52.72%, environmental 56.36%), comparable to Martínez *et al.,* (2022), who reported 50–60% in clinical isolates.

Siderophore production was more frequent in clinical isolates (54.54%) than environmental ones (34.54%), echoing findings by Lee *et al.,* (2019) and Zong *et al.,* (2018), who reported ~55–58% in clinical versus ~30–33% in environmental isolates. The higher prevalence in clinical strains underscores the role of iron acquisition in host survival and virulence.

MDR *A. baumannii* remains a formidable pathogen in ICU settings, with high resistance rates, multidrug resistance mechanisms, and substantial environmental persistence. Colistin and tigecycline retained partial activity, but emerging colistin resistance is concerning. The high prevalence of carbapenemases, particularly metallo-β-lactamases, coupled with strong biofilm-forming ability, underscores its capacity for survival and transmission.

Frequent contamination of high-touch surfaces such as beds and rails highlights the role of the environment in nosocomial spread. These findings call for reinforced infection control, targeted environmental disinfection, and prudent antibiotic stewardship to limit the impact of this resilient pathogen.

Author Contributions

Monisha B: Collected the samples, performed the preliminary and main tests, Conceptualization, Methodology, Writing - Original Draft Preparation, Visualization, Project Administration, and Funding Acquisition.; Rashmi P Mahale: Software, Validation, Formal Analysis, Writing - Review & Editing; M Raghavendra Rao: Investigation, Resources, Data Curation; Suchitra Shenoy M: Constructed the agreements and errors, Supervision.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval and Informed Consent Statement

Study Involving Identifiable Human Data or Samples - With Consent: "The research involving the use of human data/samples was approved by the Institutional Review Board of JSSAHER University (Approval No. JSS|MC|PG|0040|2022-2023|Dated 05-04-2023). Informed consent for the use of their data/samples was obtained from all participants."

Conflict of Interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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How to cite this article:

Monisha, B., Rashmi P. Mahale, Morubagal Raghavendra Rao and Suchitra Shenoy, M. 2025. Virulence Traits, Colistin Resistance and Carbapenemase Production in Multidrug-Resistant *Acinetobacter baumannii*: A Phenotypic Study from South India. *Int.J.Curr.Microbiol.App.Sci.* 14(08): 186-197.
doi: https://doi.org/10.20546/ijcmas.2025.1408.017