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TO EVALUATE IN VITRO ANTIBIOTIC SYNERGY COMBINATION TESTING BY CHECKERBOARD ASSAY ON MULTIDRUG-RESISTANT/PAN DRUG-RESISTANT ACINETOBACTER ISOLATES FROM INTENSIVE CARE UNIT

Clinical Microbiology

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ABSTRACT

Background:[1,2]Multidrug-resistant (MDR) and pan drug-resistant (PDR) Acinetobacter baumannii have emerged as major causes of hospital-acquired infections in intensive care units (ICUs). Therapeutic options are extremely limited, necessitating evaluation of combination antibiotic therapy. This study to isolate multidrug-resistant Acinetobacter spp. from ICU clinical samples, determine their antimicrobial susceptibility profiles, and evaluate in vitro antibiotic combination synergy of polymyxin B–ampicillin/sulbactam, rifampicin–ampicillin/sulbactam, and amikacin–sulbactam using the checkerboard assay**Methods:[3]**A total of 131 MDR/PDR Acinetobacter isolates obtained from ICU patients were included. Identification and antimicrobial susceptibility testing were performed using the VITEK-2 system. Synergy testing was carried out using the checkerboard microdilution method for the following combinations: rifampicin + ampicillin/sulbactam, amikacin + sulbactam, and polymyxin B + ampicillin/sulbactam. Fractional inhibitory concentration index (FICI) was calculated.**Results:**Rifampicin + ampicillin/sulbactam demonstrated the highest synergy (56.48%), followed by amikacin + sulbactam (39.69%). Polymyxin B + ampicillin/sulbactam showed predominantly indifferent interactions. Acinetobacter baumannii complex constituted 92.37% of isolates. Colistin and polymyxin B were the most effective single agents.**Conclusion:** Rifampicin- and sulbactam-based combinations show promising in-vitro synergy and may serve as potential therapeutic options for MDR/PDR Acinetobacter infections.

KEYWORDS

Acinetobacter baumannii, MDR, PDR, Checkerboard assay, Antibiotic Synergy

INTRODUCTION

Hospital-acquired infections (HAIs) represent a major global public health concern, particularly in intensive care units where critically ill patients are exposed to invasive procedures and broad-spectrum antibiotics. [1,] Among Gram-negative pathogens, *Acinetobacter baumannii* has emerged as a leading cause of ventilator-associated pneumonia, bloodstream infections, urinary tract infections, wound infections, and meningitis.[2]

The World Health Organization has classified carbapenem-resistant *A. baumannii* as a Priority critical pathogen. In India, surveillance studies report carbapenem resistance exceeding 70–85% in ICU isolates. Resistance mechanisms include β-lactamase production, efflux pump overexpression, porin loss, target site mutations, and biofilm formation.[3]

Multidrug-resistant (MDR) strains are resistant to at least one agent in three or more antimicrobial classes, while pan drug-resistant (PDR) strains are resistant to all available agents. The increasing prevalence of such strains has resulted in limited treatment options, increased mortality, prolonged hospital stays, and higher healthcare costs.[4]

Combination antibiotic therapy offers a promising approach by enhancing bactericidal activity, reducing resistance emergence, and improving outcomes. Among available in-vitro methods, the checkerboard assay is widely used for evaluating antibiotic interactions by calculating the fractional inhibitory concentration index (FICI).[5]

Therefore, the present study was undertaken to evaluate the in-vitro synergistic activity of selected antibiotic combinations against MDR and PDR *Acinetobacter* isolates from ICU patients using the checkerboard method [5]

MATERIALS AND METHODS

Study Design and Setting

A hospital-based cross-sectional study was conducted in the Department of Microbiology, Santosh Medical College and Hospital, Ghaziabad, Uttar Pradesh, India, from June 2024 to December 2025.

Study Population

All ICU patients from whom *Acinetobacter* species were isolated and classified as multidrug-resistant (MDR) or pan drug-resistant (PDR) were included in the study.

Sample Size [6]

A total of 131 MDR and PDR *Acinetobacter* isolates were analysed.

Inclusion Criteria

ICU patients aged ≥18 years
Culture positive for *Acinetobacter* spp.
MDR or PDR isolates

Exclusion Criteria

Duplicate isolates
non-ICU samples
Mixed growth cultures

Identification of Isolates [7]

Clinical samples were processed using standard microbiological techniques. Isolates were identified using the VITEK-2 Compact automated system based on biochemical reactions.

Antimicrobial Susceptibility Testing [8]

Antimicrobial susceptibility testing was performed using VITEK-2 AST cards according to CLSI 2025 guidelines. Antibiotics tested included carbapenems, aminoglycosides, fluoroquinolones, tetracyclines, polymyxins, cephalosporins, and β-lactam/β-lactamase inhibitor combinations.

Checkerboard Synergy Testing [9,10,11]

Synergy testing of MDR & PDR isolates was performed using HiMIC™ checkerboard microdilution plates.

Inoculum Preparation

Isolated colonies were suspended in sterile saline and adjusted to 0.5 McFarland standard, corresponding to approximately 1×10^6 CFU/mL.

Procedure

Two-fold serial dilutions of antibiotics were prepared in microtiter wells. Each well contained a combination of two antibiotics in varying concentrations. Plates were incubated at 35–37°C for 18–24 hours.

FICI Calculation [5]

$$FICI = \frac{MIC_{Acomb}}{MIC_{Aalone}} + \frac{MIC_{Bcomb}}{MIC_{Balone}}$$

Interpretation
FICI INTERACTION –
≤0.5 Synergy, 0.5–1 Additive, 1–4 Indifferent, >4 Antagonistic

Antibiotic Combinations Tested

Drug A	Drug B
Amikacin	Sulbactam
Polymyxin B	Ampicillin/Sulbactam
Rifampicin	Ampicillin/Sulbactam

Quality Control
ATCC reference strains were used for quality assurance
- *Escherichia coli* ATCC 25922
- *Pseudomonas aeruginosa* ATCC 27853
- *Staphylococcus aureus* ATCC 29213

Statistical Analysis
Software:
a. Microsoft Excel was be used for data compilation and entry.
b. SPSS version 20.0 or higher were be used for statistical analyses.

RESULTS

This study evaluated the in-vitro antibiotic synergy against multidrug-resistant (MDR) and pan drug-resistant (PDR) *Acinetobacter baumannii* isolates obtained from intensive care unit (ICU) patients using the checkerboard microdilution assay. A total of 131 ICU patients with confirmed MDR/PDR *Acinetobacter* infections were included. Male patients predominated (58.78%) compared to females (41.22%). The majority of patients belonged to the 21–40-year age group (42.75%), followed by those aged 41–60 years (26.72%) and 61–80 years (25.19%), indicating greater involvement of middle-aged and elderly individuals. Endotracheal aspirates were the most common clinical specimens (41.22%), followed by blood samples (30.53%), reflecting ventilator-associated and bloodstream infections as major clinical presentations. The *Acinetobacter baumannii* complex was the predominant species isolated (92.37%), confirming its significant role as a nosocomial ICU pathogen.

Antimicrobial susceptibility testing revealed the highest sensitivity to polymyxins (23.4%), with colistin (40.9%) .In contrast, extremely poor susceptibility was observed for carbapenems, aminoglycosides, fluoroquinolones, and β-lactam antibiotics, confirming the extensive drug-resistant nature of the isolates in VITEK-2. Risk factor analysis demonstrated that prior antimicrobial exposure was the most frequent predisposing factor, present in 84.73% of patients. Advanced age (>55 years) was observed in 62.6% of cases, while mechanical ventilation was documented in 61.83% of patients, highlighting its importance as a major risk factor for ICU-acquired infections. Invasive procedures, including central venous catheterization and other ICU interventions, were noted in 44.27% of cases, and comorbidities such as diabetes mellitus, chronic kidney disease, and cardiovascular disorders were present in 38.93% of patients. Overall, most patients had multiple concurrent risk factors, with the majority exhibiting three or more risk factors and 38.17% having four risk factors, emphasizing the multifactorial nature of MDR/PDR *A. baumannii* infections.

The total combination of risk factor probability according to which severity predication has been develop, Risk Matrix probability with 25 Risk-Factor Combinations

FIGURE:1 COMBINATION INDICATING RISK FACTOR PROBABILITY

123 (74%)	124 (66%)	134 (66%)	125 (62%)	135 (62%)
234 (59.2%)	12 (51%)	13 (51%)	145 (55%)	235 (55%)
14 (44%)	23 (44%)	245 (48%)	345 (48%)	15 (40%)
24 (37%)	34 (37%)	35 (33%)	25 (26%)	45 (26%)
1 (29%)	2 (22%)	3 (22%)	4 (15%)	5 (11%)

1. Prior antimicrobial exposure: 84.73% (Value = 8)
2. Age >55yrs: 62.6% (Value = 6)
3. Mechanical ventilation: 61.83% (Value = 6)
4. Invasive procedures: 44.27% (Value = 4)
5. Comorbidities: 38.93% (Value = 3)

Total Value from risk factor variables (1 to 5) is 27

Severe (70-80%), Moderate (60-70%), Mild (50-60%) Low (<50%)

In-vitro synergy testing demonstrated that the combination of rifampicin with ampicillin/sulbactam exhibited the highest synergistic activity (56.48%). The combination of amikacin with sulbactam showed moderate synergy or additive effects (39.69%), while polymyxin B combined with ampicillin/sulbactam was largely indifferent and, in some instances, antagonistic.

TABLE:1: CHECKERBOARD SYNERGY RESULTS FOR ANTIBIOTIC COMBINATIONS AGAINST MDR/PDR ACINETOBACTER ISOLATES.

Antibiotics	Additivity (n)	Antagonism (n)	Indifference (n)	Synergy (n)	Synergy (%)
Amikacin + Sulbactam	55	0	24	52	39.69%
Polymyxin B + Ampicillin/ Sulbactam	34	28	69	0	0.00%
Rifampicin + Ampicillin/ Sulbactam	40	0	17	74	56.48%

Polymyxin B showed the highest overall sensitivity (89.31%), whereas the highest resistance rates were observed with ampicillin/sulbactam (68.70%) and amikacin (63.36%).

TABLE:2 ANTIBIOGRAM SUMMARY OF MDR/PDR ACINETOBACTER ISOLATES.

Antibiotic	Resistant (n)	Sensitive (n)	*Resistance (%)*	Sensitivity (%)
Polymyxin B	**14**	**117**	**10.69%**	**89.31%**
Rifampicin	**56**	**75**	**42.75%**	**57.25%**
Ampicillin/ Sulbactam	**90**	**41**	**68.70%**	**31.30%**
Amikacin	**83**	**48**	**63.36%**	**36.64%**
Sulbactam	**71**	**60**	**54.20%**	**45.80%**

Notably, synergy was observed even among isolates resistant to individual drugs, with amikacin-resistant and sulbactam-resistant isolates showing synergy in 44.23% of cases, and rifampicin-resistant and ampicillin/sulbactam-resistant isolates showing synergy in 50% of cases. Rifampicin-based combinations consistently demonstrated superior synergistic activity.

TABLE:3 INDIVIDUAL DRUG RESISTANCE (R) IN SYNERGY ISOLATES.

SYNERGY ISOLATES	DRUG A IN SYNERGY ISOLATES (R)	DRUG B IN SYNERGY ISOLATES (R)	DRUG A & B IN SYNERGY ISOLATES BOTH (R)	PERCENTAGEOF SYNERGY INA &B DRUG BOTH (R)
Amikacin (DRUG A) + sulbactam (DRUG B) (52)	**37**	**45**	**23**	**44.23%**
Rifampicin (DRUG A) + ampicillin/ sulbactam (DRUG B) (74)	**53**	**42**	**37**	**50.00%**

Key Observations
Rifampicin + ampicillin/sulbactam showed highest synergy.
Amikacin+ Sulbactam-based combinations were more effective.
Polymyxin B+ Sulbactam combinations showed predominantly indifferent effects.

DISCUSSION

The most striking result of the present study is the high frequency of synergy observed with Rifampicin + Ampicillin/Sulbactam, with more than half of the tested isolates demonstrating synergistic interaction and no antagonism. This finding is strongly supported by prior

literature. Cetin et al. demonstrated that Rifampicin combined with Ampicillin/Sulbactam or Cefoperazone/Sulbactam showed exclusive synergistic activity, whereas polymyxin-based sulbactam combinations were largely indifferent or antagonistic [5]. Similar observations were reported by Ozseven et al., where Rifampicin-containing combinations showed higher synergy rates compared to polymyxin-based regimens [26]. The mechanistic rationale for this synergy lies in Rifampicin's inhibition of bacterial RNA polymerase, which enhances intracellular antibiotic penetration when the bacterial cell wall is destabilized by sulbactam's intrinsic anti-*Acinetobacter* activity.

The Amikacin + Sulbactam combination demonstrated moderate synergy and additivity in the present study, consistent with findings reported by Halim et al., who observed synergistic effects in over half of MDR isolates tested with this combination using checkerboard assays [31]. Sulbactam's affinity for penicillin-binding proteins in *A. baumannii* facilitates enhanced aminoglycoside uptake, thereby explaining the observed interaction. However, variability in synergy across isolates highlights strain-dependent resistance mechanisms, particularly efflux pump activity and aminoglycoside-modifying enzymes [3].

In contrast, Polymyxin B + Ampicillin/Sulbactam showed predominantly indifferent interactions, with occasional antagonism. While polymyxins remain essential salvage agents, their combination efficacy has been inconsistent across studies. Lee et al. reported largely indifferent effects for colistin-based combinations using checkerboard testing [14], and Tan et al. demonstrated substantial discordance between checkerboard, time-kill, and E-test methods for polymyxin-based regimens [15]. Although other investigators have reported synergy with polymyxin combinations, particularly when assessed by time-kill assays or triple-drug regimens [16,17], the present findings suggest that polymyxin B may not consistently enhance sulbactam activity in vitro when evaluated by checkerboard methodology alone.

CONCLUSION

Rifampicin combined with ampicillin/sulbactam demonstrated the highest in-vitro synergistic activity against MDR and PDR *Acinetobacter* isolates. Amikacin with sulbactam also showed promising results. These findings support the potential clinical utility of sulbactam-based combinations in the management of resistant *Acinetobacter* infections.

This research emphasis the need for synergy testing in MDR&PDR isolates on case to case basis and from the result of above synergy testing a unique insight is reflected is that approx. 45% cases of synergistic result in vividly both drug were resistance. Hence there pressing need for conducting research on the basis for selection of antibiotics for synergy testing

Further clinical trials are recommended to validate these in-vitro observations.

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