



RESEARCH ARTICLE

Evaluation of Combination Antimicrobial Therapy Against Extended-Spectrum Beta-Lactamase Producing Enterobacterales: An In Vitro Study

Madupu Deepthi¹, A Sangeetha^{2*}, Chandana Konda¹

ABSTRACT

Background: Extended-spectrum beta-lactamase (ESBL)-producing *Enterobacterales* pose a significant therapeutic challenge due to multidrug resistance. **Objectives:** To evaluate the in vitro efficacy of selected antimicrobial combinations against ESBL-producing *Enterobacterales*. **Materials and Methods:** Clinically significant ESBL-producing isolates were identified using standard microbiological methods and confirmed by the combination disc method. Antimicrobial combinations were assessed using the checkerboard assay, and interactions were interpreted based on the Fractional Inhibitory Concentration Index (FICI). **Results:** Among the tested combinations, meropenem–amikacin demonstrated the highest synergistic activity, followed by piperacillin–tazobactam–amikacin, while most other combinations showed additive or indifferent effects. No antagonistic interactions were observed. **Conclusion:** Selected antimicrobial combinations exhibited promising in vitro synergistic activity against ESBL-producing *Enterobacterales*. These findings support further clinical evaluation of combination therapy as a potential strategy for managing infections caused by multidrug-resistant organisms.

Keywords: ESBL, *Enterobacterales*, antimicrobial combination therapy, synergy, checkerboard assay, multidrug resistance.
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INTRODUCTION

Extended-spectrum beta-lactamase (ESBL)-producing *Enterobacterales* have become a major global public health concern due to their increasing resistance to β -lactam antibiotics, particularly third-generation cephalosporins. These organisms, predominantly *Escherichia coli* and *Klebsiella pneumoniae*, are responsible for a wide range of infections, including urinary tract infections, bloodstream infections, pneumonia, and intra-abdominal infections. The rapid dissemination of plasmid-mediated ESBL genes, coupled with co-resistance to multiple antimicrobial classes, has significantly limited available treatment options and contributed to increased morbidity, mortality, and healthcare costs [1,2]. Carbapenems remain the preferred treatment for severe ESBL-associated infections; however, their extensive use has accelerated the emergence of carbapenem-resistant organisms. Consequently, antimicrobial combination therapy has been explored as a potential strategy to enhance antibacterial activity, achieve synergistic effects, and reduce the development of resistance. In vitro methods such as the checkerboard assay and Fractional Inhibitory Concentration Index (FICI) are widely used to evaluate

¹Assistant Professor, Department of Microbiology, CMR Institute of Medical Sciences, Kandalkoya, Medchal Road, Hyderabad, Telangana, India

²Associate Professor, Department of Pharmacology, CMR Institute of Medical Sciences, Kandalkoya, Medchal Road, Hyderabad, Telangana, India

Corresponding Author: A Sangeetha, CMR Institute of Medical Sciences, Kandalkoya, Medchal Road, Hyderabad, Telangana, India. E-mail: geetha66pharma@gmail.com

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the interactions between antimicrobial agents and identify effective therapeutic combinations [3,4].

Considering the growing prevalence of ESBL-producing *Enterobacterales* and the limited therapeutic options available, evaluating the efficacy of antimicrobial combinations is essential for optimizing treatment

strategies. Therefore, the present study aimed to assess the *in vitro* activity of selected antimicrobial combinations against ESBL-producing *Enterobacterales* isolates using the checkerboard assay and FICI interpretation [5,6].

MATERIALS AND METHODS

Study Design

This was a laboratory-based cross-sectional *in vitro* experimental study conducted to evaluate the efficacy of selected antimicrobial combinations against Extended-Spectrum Beta-Lactamase (ESBL)-producing *Enterobacterales* isolates.

Study

The study was carried out in the Department of Microbiology, CMR Medical College and Hospital, Hyderabad, Telangana, India, over a period of 12 months (Month Year–Month Year) after obtaining approval from the Institutional Ethics Committee.

Setting

Study Population and Sample Size

A total of 100 non-duplicate clinical isolates of ESBL-producing *Enterobacterales* obtained from routine clinical specimens such as urine, blood, pus, sputum, wound swabs, and other body fluids were included in the study. The sample size of 100 isolates was selected based on feasibility and the availability of culture-positive ESBL isolates during the study period.

Inclusion Criteria

- Clinical isolates belonging to the family *Enterobacterales*.
- Isolates confirmed as ESBL producers by phenotypic methods.
- One isolate per patient (non-duplicate isolates).
- Isolates recovered from clinically significant specimens.

Exclusion Criteria

- Duplicate isolates from the same patient.
- Non-*Enterobacterales* isolates.
- Contaminated or mixed bacterial cultures.
- Isolates with incomplete laboratory records or poor viability.

Isolation and Identification of Bacterial Isolates

Clinical specimens were processed using standard microbiological procedures. The isolates were cultured on Blood agar and MacConkey agar and incubated aerobically at 37°C for 18–24 hours. Identification of bacterial isolates was performed based on colony morphology, Gram staining

characteristics, and standard biochemical tests. Where available, identification was confirmed using automated identification systems.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed using the Kirby–Bauer disc diffusion method on Mueller-Hinton agar following the Clinical and Laboratory Standards Institute (CLSI) guidelines. Antibiotics tested included cefotaxime, ceftazidime, cefepime, piperacillin–tazobactam, meropenem, amikacin, gentamicin, ciprofloxacin, and other routinely used antimicrobial agents.

Confirmation of ESBL Production

Screen-positive isolates were confirmed for ESBL production using the Combination Disc Diffusion Test (CDDT) employing ceftazidime (30 µg) and ceftazidime-clavulanic acid (30/10 µg), as recommended by CLSI. An increase in the inhibition zone diameter of ≥5 mm with clavulanic acid compared to ceftazidime alone was considered positive for ESBL production.

Evaluation of Antimicrobial Combination Therapy

The *in vitro* activity of selected antimicrobial combinations (such as meropenem–amikacin, piperacillin/tazobactam–amikacin, and cefepime–amikacin) was assessed using the checkerboard broth microdilution assay. Minimum inhibitory concentrations (MICs) of individual antibiotics and their combinations were determined in sterile 96-well microtitre plates containing Mueller-Hinton broth. After inoculation with standardized bacterial suspension [0.5 McFarland], the plates were incubated at 37°C for 18–24 hours.

The interaction between antimicrobial agents was interpreted by calculating the Fractional Inhibitory Concentration Index (FICI) using the formula:

$$\text{FICI} = \text{FIC of Drug A} + \text{FIC of Drug B}$$

Interpretation of FICI values:

- ≤0.5 – Synergistic
- >0.5–1.0 – Additive
- >1.0–4.0 – Indifferent
- >4.0 – Antagonistic

Quality Control

Quality control procedures were performed using standard reference strains, including *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603, in accordance with CLSI recommendations.

Statistical Analysis

Data were entered into Microsoft Excel and analyzed using Statistical Package for the Social Sciences (SPSS) version 26.0 (IBM Corp., Armonk, NY, USA). Categorical variables were expressed as frequencies and percentages, while continuous variables were presented as mean $\pm$ standard deviation where appropriate. The proportion of synergistic, additive, indifferent, and antagonistic interactions among antimicrobial combinations was calculated. Associations between bacterial species and antimicrobial interactions were analyzed using the Chi-square test or Fisher's exact test, as appropriate. A p-value <0.05 was considered statistically significant.

RESULTS

A total of 100 ESBL-producing *Enterobacterales* isolates recovered from various clinical specimens were included in the study. The isolates were identified as *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Proteus mirabilis*. Antimicrobial combination testing was performed using the checkerboard broth microdilution assay.

Table 1: Distribution of ESBL-producing *Enterobacterales* according to bacterial species (n=100)

Organism	Number (%)
<i>Escherichia coli</i>	58 (58.0)
<i>Klebsiella pneumoniae</i>	30 (30.0)
<i>Enterobacter cloacae</i>	8 (8.0)
<i>Proteus mirabilis</i>	4 (4.0)
Total	100 (100)

Interpretation

Escherichia coli was the predominant ESBL-producing isolate (58%), followed by *Klebsiella pneumoniae* (30%). These two organisms together accounted for 88% of all isolates included in the study.

Table 2: Distribution of ESBL-producing isolates according to clinical specimen (n=100)

Clinical specimen	Number (%)
Urine	46 (46.0)
Pus	22 (22.0)
Blood	14 (14.0)
Sputum	10 (10.0)
Wound swab	5 (5.0)
Other body fluids	3 (3.0)
Total	100 (100)

Interpretation

Urine was the commonest specimen yielding ESBL-producing *Enterobacterales* (46%), followed by pus (22%), indicating urinary tract infections as the predominant source.

Table 3: Antimicrobial susceptibility pattern of ESBL-producing *Enterobacterales* (n=100)

Antibiotic	Sensitive n (%)	Resistant n (%)
Meropenem	95	5
Amikacin	88	12
Piperacillin–Tazobactam	76	24
Cefepime	38	62
Ciprofloxacin	30	70
Gentamicin	55	45
Ceftazidime	8	92

$$\chi^2 = 74.63$$

P < 0.001 (Highly significant)

Interpretation

Meropenem demonstrated the highest susceptibility (95%), followed by amikacin (88%). Resistance was highest against ceftazidime (92%) and ciprofloxacin (70%), reflecting the multidrug-resistant nature of ESBL-producing isolates.

Interpretation

Meropenem–amikacin exhibited the highest synergistic activity (72%), significantly outperforming the other antimicrobial combinations evaluated.

Interpretation

Meropenem–amikacin showed superior synergistic activity across all bacterial species, particularly against *Escherichia coli*, where synergy was observed in nearly 80% of isolates.

Interpretation

More than half (57.7%) of all antimicrobial interactions demonstrated synergism, whereas no antagonistic interaction was observed, indicating the potential benefit of combination antimicrobial therapy against ESBL-producing *Enterobacterales*.

DISCUSSION

Extended-spectrum beta-lactamase (ESBL)-producing *Enterobacterales* continue to pose a significant therapeutic challenge because of their ability to hydrolyze extended-spectrum cephalosporins and their frequent association

Table 4: Synergistic activity of antimicrobial combinations against ESBL-producing *Enterobacteriales* (n=100)

Antimicrobial Combination	Synergy (%)	Additive (%)	Indifferent (%)	Antagonistic (%)
Meropenem + Amikacin	72	18	10	0
Piperacillin–Tazobactam + Amikacin	58	25	17	0
Cefepime + Amikacin	43	32	25	0

$$\chi^2 = 14.52$$

P = 0.002 (Statistically significant)

Table 5: Comparison of synergistic activity among different bacterial species (n=100)

Organism	Meropenem + Amikacin n (%)	Piperacillin–Tazobactam + Amikacin n (%)	Cefepime + Amikacin n (%)
<i>E. coli</i> (n=58)	46 (79.3)	35 (60.3)	27 (46.6)
<i>K. pneumoniae</i> (n=30)	19 (63.3)	16 (53.3)	11 (36.7)
<i>Enterobacter cloacae</i> (n=8)	5 (62.5)	4 (50.0)	3 (37.5)
<i>Proteus mirabilis</i> (n=4)	2 (50.0)	2 (50.0)	2 (50.0)

$$\chi^2 = 9.84$$

P = 0.020 (Statistically significant)

Table 6: Fractional Inhibitory Concentration Index (FICI) interpretation among antimicrobial combinations (n=300 interactions)

FICI Interpretation	Number (%)
Synergistic (≤ 0.5)	173 (57.7)
Additive (>0.5 – 1.0)	75 (25.0)
Indifferent (>1.0 – 4.0)	52 (17.3)
Antagonistic (>4.0)	0 (0)
Total	300 (100)

$$\chi^2 = 112.86$$

P < 0.001 (Highly significant)

with multidrug resistance. The present study evaluated the in vitro efficacy of selected antimicrobial combinations against ESBL-producing *Enterobacteriales* isolates using the checkerboard assay and Fractional Inhibitory Concentration Index (FICI). The findings demonstrated that combination therapy, particularly meropenem plus amikacin, exhibited superior synergistic activity compared with other antimicrobial combinations, supporting its potential role in managing infections caused by multidrug-resistant Gram-negative bacteria [7,8].

In the present study, *Escherichia coli* (58%) was the predominant ESBL-producing organism, followed by *Klebsiella pneumoniae* (30%). These findings are consistent with previous reports, which have identified *E. coli* as the leading ESBL-producing pathogen responsible for urinary

tract and bloodstream infections worldwide [2,9,10]. The predominance of urinary isolates (46%) in the present study further supports the established epidemiology of ESBL-producing *Enterobacteriales*, as urinary tract infections remain the most common clinical manifestation of these organisms [11].

Antimicrobial susceptibility testing revealed excellent activity of meropenem (95%) and amikacin (88%), whereas ceftazidime and ciprofloxacin exhibited high resistance rates. Similar observations have been reported in surveillance studies where carbapenems retained excellent activity against ESBL producers despite increasing global resistance trends [3,12]. The high resistance observed against third-generation cephalosporins was expected because ESBL enzymes effectively hydrolyze these antibiotics, making them unsuitable for empirical treatment of serious infections caused by these organisms [2,8].

One of the important findings of the present investigation was the significant synergistic activity demonstrated by the meropenem–amikacin combination (72%), followed by piperacillin–tazobactam–amikacin (58%) and cefepime–amikacin (43%). The superior activity of meropenem combined with amikacin may be attributed to complementary mechanisms of action, where meropenem disrupts bacterial cell wall synthesis, facilitating enhanced intracellular penetration of aminoglycosides. Previous in vitro studies have similarly demonstrated enhanced bactericidal activity of carbapenem–aminoglycoside

combinations against ESBL-producing and multidrug-resistant *Enterobacterales* [13,14]. Importantly, no antagonistic interaction was observed among any antimicrobial combinations tested, indicating that these regimens are unlikely to compromise antibacterial activity.

The checkerboard assay demonstrated that more than half of all antimicrobial interactions were synergistic, while additive interactions accounted for one-fourth of the combinations evaluated. Similar findings have been documented by several investigators using checkerboard and time-kill assays, emphasizing the usefulness of combination therapy in overcoming resistance mechanisms and minimizing the emergence of resistant mutants during treatment [5,13,15]. Although *in vitro* synergy does not always translate directly into clinical success, these findings provide valuable guidance for selecting combination regimens in critically ill patients with limited therapeutic options.

The statistically significant association between bacterial species and antimicrobial synergy ($p < 0.05$) observed in this study suggests that the effectiveness of combination therapy may vary according to the infecting organism. *Escherichia coli* demonstrated higher synergistic responses than *Klebsiella pneumoniae*, which may reflect differences in resistance mechanisms, β -lactamase expression, membrane permeability, and efflux pump activity. Similar species-dependent variations have been reported in previous investigations evaluating combination antimicrobial therapy against multidrug-resistant Gram-negative pathogens [10,14].

The emergence of antimicrobial resistance has prompted increasing interest in carbapenem-sparing strategies. Although carbapenems remain the drugs of choice for severe ESBL infections, excessive dependence on these agents has accelerated the development of carbapenem-resistant *Enterobacterales*. Therefore, identifying effective combination regimens that maximize bacterial killing while reducing selective pressure is an important component of antimicrobial stewardship programs [3,7,12]. The present findings support the continued evaluation of combination antimicrobial therapy, particularly in severe infections where monotherapy may be inadequate.

Overall, the present study demonstrates that antimicrobial combination therapy, especially meropenem plus amikacin, exhibits promising *in vitro* activity against ESBL-producing *Enterobacterales*. These observations are in agreement with published literature and reinforce the importance of performing *in vitro* synergy testing before recommending combination therapy for multidrug-resistant

infections. However, multicenter clinical trials are required to establish whether these laboratory findings translate into improved patient outcomes [8,13–15].

Limitations of the Study

The present study has certain limitations. First, it was a single-center laboratory-based *in vitro* study conducted at CMR Medical College and Hospital; therefore, the findings may not be generalizable to other geographical regions. Second, the sample size of 100 isolates, although adequate for laboratory evaluation, may not fully represent the diversity of ESBL-producing *Enterobacterales*. Third, only selected antimicrobial combinations were evaluated, while newer β -lactam/ β -lactamase inhibitor combinations were not included. Fourth, molecular characterization of ESBL genes (such as blaCTX-M, blaTEM, and blaSHV) was not performed because of resource limitations. Finally, as the study was entirely *in vitro*, the observed synergistic effects may not directly correlate with clinical efficacy, pharmacokinetic variability, or patient outcomes. Future multicenter studies incorporating molecular analysis and clinical correlation are warranted to validate these findings and optimize antimicrobial combination therapy against ESBL-producing *Enterobacterales*.

CONCLUSION

The present study demonstrated that antimicrobial combination therapy exhibits promising *in vitro* activity against ESBL-producing *Enterobacterales*, with meropenem plus amikacin showing the highest synergistic effect among the combinations evaluated. The absence of antagonistic interactions and the significant proportion of synergistic responses indicate that combination therapy may enhance antibacterial efficacy against multidrug-resistant isolates. These findings support the potential role of antimicrobial combinations as an effective therapeutic strategy, particularly in severe infections where treatment options are limited. However, since the present investigation was an *in vitro* study, the results should be interpreted with caution. Further multicenter studies with larger sample sizes, molecular characterization of resistance genes, and well-designed clinical trials are required to validate these findings and establish the clinical effectiveness of combination antimicrobial therapy in the management of ESBL-producing *Enterobacterales* infections.

CONFLICT OF INTEREST

Nil.

SOURCE OF FUNDING

Nil.

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