JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

A RAPID SCREENING PROTOCOL FOR DETECTING PHARMACOLOGICAL INTERACTIONS: A NOVEL APPROACH TO MITIGATE MULTIDRUG RESISTANCE

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https://doi.org/10.55251/jmbfs.12784

ARTICLE INFO

Received 9. 5. 2025
Revised 26. 6. 2025
Accepted 3. 7. 2025
Published 1. 8. 2025

Regular article

OPEN ACCESS

ABSTRACT

The challenge of antimicrobial resistance (AMR) underscores the complexities in managing multidrug-resistant (MDR) infections. This is escalating the need for straightforward and practical strategies to assess antibiotic interactions. The present study aimed to establish a rapid screening method for the identification of drug interactions using the disc diffusion (DD) technique, which was validated against the time-kill assay (TKA). A total of 100 bacterial isolates, including *Acinetobacter* spp., *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella* spp., were assessed on Mueller-Hinton Agar (MHA) plates with antibiotics at various concentrations. The DD method identified synergistic interactions in 58 isolates, of which 55% (32 isolates) were confirmed by TKA, showing moderate concordance between the two methods. Similarly, among the 25 isolates exhibiting antagonistic effects as determined by DD, 56% (14 isolates) were validated through TKA. The findings highlight the reliability of the DD method as a simplified, low-cost preliminary screening tool for detecting antibiotic interactions, capable of delivering results as early as within 24 hours. This DD method thus presents a valuable instrument for identifying effective drug combinations in MDR infections, providing rapid and reliable results suitable for critical care environments, particularly in resource-limited settings. However, additional research incorporating more antibiotics and bacterial species is strongly recommended to validate these findings, not only *in vitro* but also *in vivo*. Integrating molecular techniques with the DD method could lead to a more targeted therapeutic approach and improved management of symptoms, particularly in the cases of MDR.

Keywords: Disc diffusion method, Time-kill assay, *Acinetobacter* spp., *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella* spp., synergism, antagonism

INTRODUCTION

Antimicrobial resistance (AMR) has emerged as a significant global health threat, not only complicating the treatment of infectious diseases ***(Hossain et al., 2024)*** but also leading to the failure of even the most advanced antibiotics ***(Catalano et al., 2022)***. The World Health Organization (WHO, 2022) already warned that antibiotic resistance has reached alarming levels globally, resulting in increased morbidity and mortality ***(Gajic et al., 2022)***. In 2019, six significant pathogens, *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, were reported as responsible for 929,000 deaths linked directly to AMR and 3.57 million deaths associated with it – which could reach 10 million by 2050 ***(Gajic et al., 2022)***. Recently, ***Naghavi et al. (2024)*** projected that AMR would cause 1.91 million deaths annually by 2050, with 8.22 million deaths associated with it, resulting in a cumulative burden of 39.1 million attributable and 169 million associated deaths between 2025 and 2050. Key pathogens, such as *S. aureus* and the carbapenem-resistant *A. baumannii-calcoaceticus* (CRAB) complex, pose significant challenges in community settings in this regard ***(Jeannot et al., 2023; Kang et al., 2023)***.

However, combined antimicrobial remedy presents a promising approach against MDR infections, potentially extending the lifespan of antimicrobials and reducing the development of resistance ***(Giamarellou et al., 1986; Gaudereto et al., 2020)***. Traditional methods for assessing synergy, such as the time-kill assay (TKA) and checkerboard assay (CBA), although considered standards, are relatively expensive, time-consuming, and require technical expertise ***(Chinwuba et al., 1991; Balouiri et al., 2016)***.

The increasing prevalence of AMR has predominantly been attributed to the extensive and frequently improper application of antibiotics not only within the domains of human healthcare but also agriculture and allied sectors ***(Omoya et al., 2016)***. The overuse of antibiotics, particularly third- and fourth-generation cephalosporins, along with poor patient compliance, has significantly contributed to the global antibiotic resistance crisis ***(Yadav et al., 2016)***. Although this problem is acute in intensive care units (ICUs), where multidrug-resistant (MDR) infections have become a substantial public health challenge ***(Fadwa et al., 2021)***. This issue is exacerbated further by a lack of new antibiotic development and by inappropriate prescribing practices, resulting in the emergence of resistant strains within *Enterobacteriaceae* and other pathogens worldwide ***(Yadav et al., 2016; Alrashidi et al., 2021)***. The coronavirus disease-2019 (COVID-19) pandemic has intensified the AMR crisis in recent times, primarily through the heightened and frequent improper use of antibiotics for viral infections. This situation highlights the critical need for enhanced antimicrobial stewardship measures ***(Gajic et al., 2022)***. The resulting resistance to last-resort antibiotics, such as colistin and carbapenems, highlights the urgent need to reevaluate existing drugs and develop new therapeutic strategies ***(Antonello et al., 2020; Fadwa et al., 2021)***. Furthermore, reducing the turnaround time for testing antimicrobial efficacy has become crucial for lowering morbidity, mortality, and healthcare costs ***(Gaudereto et al., 2020)***. Therefore, the present study aimed to establish a simple, rapid, and low-cost screening technique for identifying interactive antibiotic combinations using the DD method. This new approach aims to provide a faster and more effective method for assessing drug interactions, particularly in middle- and low-income countries.

MATERIALS AND METHODS

Bacterial Strains

This investigation utilized a comprehensive experimental design to develop and validate an efficient screening method for detecting drug interactions in MDR bacterial strains: *Acinetobacter* spp., *Escherichia coli*, *Pseudomonas aeruginosa,* and *Klebsiella* spp. These pathogens are widely recognized for their well-documented resistance profiles. The isolates were obtained from urine, pus, sputum, and bronchoalveolar lavage fluid (BALF) samples collected at a research laboratory based in Kolkata, India.

Sample Preparation

Individual bacterial strains were lawn cultured on Mueller Hinton Agar (MHA) plates, with each strain inoculated onto three separate MHA plates under different conditions: a control plate with no antibiotic, a plate containing 50% minimum inhibitory concentration (MIC) of ceftriaxone (CTR), and a plate with 50% MIC of meropenem (MRP).

Antibiotic Susceptibility Testing

Modified Kirby-Bauer Disc Diffusion Method

DD method, which has been the standard antimicrobial susceptibility testing (AST) since its development in 1940, was employed for this study due to its flexibility, cost-effectiveness, and reliability in microbiology laboratories ***(Balouiri et al., 2016; Gajic, 2022)***. This method, as validated by ***Bauer et al. (1966)***, is a reliable technique for detecting antibiotic resistance, including methicillin-resistant *S. aureus* (MRSA), particularly when incubated at 35°C ***(Drew et al., 1972)***. To enhance the accuracy of their findings regarding drug interactions in the present study, strict identification protocols were put in place to confirm resistant colonies within zones of inhibition before reporting results. This methodological rigor ensured that the assessments of antibiotic interactions were based on accurately identified isolates, thereby strengthening the reliability of the current study. Solid media containing 50% of the MIC of either CTR or MRP, or antibiotic-free agar (control plate), was used as the base. This was overlaid with the bacterial inoculum, followed by the placement of antimicrobial discs, and incubated at 37°C for observation of results ***(Chinwuba et al., 1991; Zafar et al., 2015; Laishram et al., 2017)***. The discs included tigecycline (TGC, 15 µg), chloramphenicol (C, 30 µg), cotrimoxazole (COT, 25 µg), polymyxin B (PB, 300 µg), azithromycin (AZM, 15 µg), imipenem (IPM, 10 µg), aztreonam (AT, 30µg), cefoperazone (CPZ, 75 µg), lomefloxacin (LOM 10, µg), meropenem (MRP, 10 µg), levofloxacin (LE, 5 µg) and amikacin (AK, 30 µg). This method assessed the antimicrobial effectiveness of drug combinations by measuring the inhibition zone diameter (IZD) formed ***(Hossain et al., 2024)***. Synergy was defined as an increase in the IZD by ≥19% when using agar containing 50% MIC of antibiotics, while indifference was indicated by no change in diameter. An increase of <19% in IZD was considered an additive effect ***(Chinwuba et al., 1991; Laishram et al., 2017)***. Antagonism was characterized by a decrease in the IZD ***(Chinwuba et al., 1991)***.

Time-Kill Assay

TKA is a well-standardized method for determining the bactericidal effect of antimicrobial agents, providing crucial insights into time- and concentration-dependent interactions between bacterial strains and antibiotics ***(Balouiri et al., 2016)***. The assay evaluates antimicrobial activity by exposing microorganisms to varying antibiotic concentrations to determine if the effect is time- or concentration-dependent ***(Hossain et al., 2024)***. Antibiotics were tested alone and in combination at a concentration of 50% MIC ***(Bozkurt-Guzel et al., 2012; Gaudereto et al., 2020; Ju et al., 2022; Kang et al., 2023)*** of CTR or MRP. Viable colony counts were performed at specific time intervals (0, 2, 4, 6, and 24 hours) to monitor the bacterial growth ***(Gaudereto et al., 2020)***. Bactericidal activity was defined as a reduction of ≥3 $\log_{10}$ colony forming units (CFU)/ml from the initial count ***(Korten et al., 1996; Messick et al., 1999; Bozkurt-Guzel et al., 2012; Laishram et al., 2017; Ju et al., 2022; Kang et al., 2023)***. Synergy and antagonism were defined as a ≥2 $\log_{10}$ CFU/ml reduction or increase, respectively, compared to the most effective single agent, while no difference was defined as a change of <2 $\log_{10}$ in colony count when using an antibiotic combination compared to individual agents ***(Gaudereto et al., 2020; Fadwa et al., 2021)***.

Data Collection

Growth inhibition was analyzed using the Kirby-Bauer method, where zones of inhibition were measured and recorded according to standard guidelines (CLSI, 2020). CFU counts from the TKA were recorded at each time point to assess bacterial viability.

RESULTS

A total of 100 MDR bacterial isolates were subjected to testing, highlighting the varied effects of antibiotic combinations utilized in the study. Indifferent effects were observed in 17% of the isolates, as no significant alterations in the diameters of the inhibition zones were noted (Figures 1A and 2A). Synergistic interactions were identified in 58% of the isolates, characterized by a >19% increase in the IZD when antibiotics were combined at 50% MIC of CTR (Figure 1B). Antagonistic effects were observed in 25% of the isolates, indicated by a decrease in IZD (Figure 2B). These findings were further supported by the results of the TKA (Figures 1C and 2C).

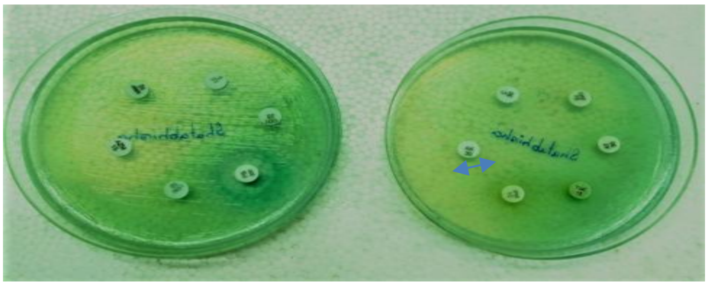

Figure 1(A) Mueller-Hinton Agar (MHA) plates without antibiotics that served as control wherein the bacterial growth is evenly distributed with limited zones of inhibition around the antibiotic discs (tigecycline - TGC, 15 µg; chloramphenicol - C, 30 µg; cotrimoxazole - COT, 25 µg; polymyxin B - PB, 300 µg; azithromycin - AZM, 15 µg; imipenem - IPM, 10 µg; aztreonam - AT, 30µg; cefoperazone - CPZ, 75 µg; lomefloxacin - LOM 10, µg; meropenem – MRP, 10 µg; levofloxacin - LE, 5 µg; and amikacin - AK, 30 µg).

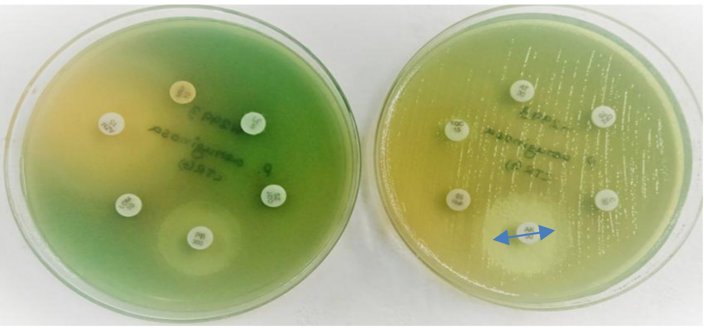

Figure 1(B) Mueller-Hinton Agar (MHA) plates containing 50% of minimum inhibitory concentration (MIC) of ceftriaxone (CTR) that show a marked increase in the zone of inhibition (indicated by the arrow) around the antibiotic discs (tigecycline - TGC, 15 µg; chloramphenicol - C, 30 µg; cotrimoxazole - COT, 25 µg; polymyxin B - PB, 300 µg; azithromycin - AZM, 15 µg; imipenem - IPM, 10 µg; aztreonam - AT, 30µg; cefoperazone - CPZ, 75 µg; lomefloxacin - LOM 10, µg; meropenem – MRP, 10 µg; levofloxacin - LE, 5 µg; and amikacin - AK, 30 µg), suggesting a synergistic interaction between the antibiotics.

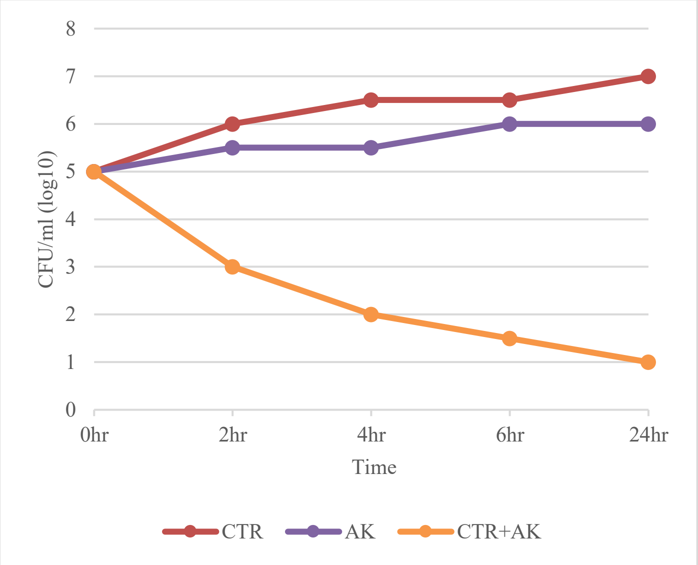

Figure 1(C) Bacterial kill kinetics of *Pseudomonas aeruginosa* for the tested combinations of antibiotics over a 24-hour period. Time-Kill Assay (TKA) results depict bacterial CFU/ml ($\log_{10}$) over time. The combination of ceftriaxone (CTR) and amikacin (AK) showed a significant reduction in bacterial count compared to either antibiotic alone, confirming a synergistic effect.

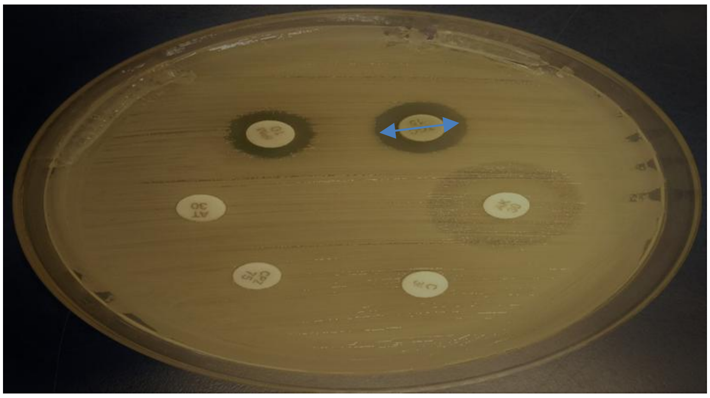

Figure 2(A) *Escherichia coli* grown on Mueller-Hinton Agar (MHA) plates without antibiotics, displaying clear inhibition zones.

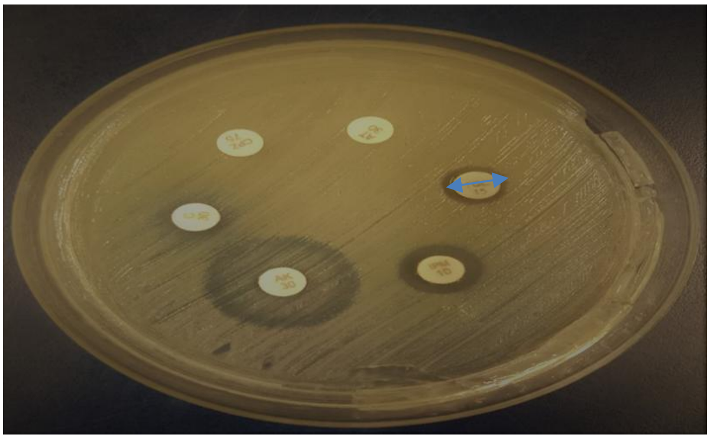

Figure 2(B) Mueller-Hinton Agar (MHA) plates containing 50% minimum inhibitory concentration (MIC) of ceftriaxone (CTR), where a marked reduction in the inhibition zone diameter (IZD) indicates antagonism compared to the individual antibiotics (tigecycline - TGC, 15 µg; chloramphenicol - C, 30 µg; imipenem - IPM, 10 µg; aztreonam - AT, 30µg; cefoperazone - CPZ, 75 µg; and amikacin - AK, 30 µg).

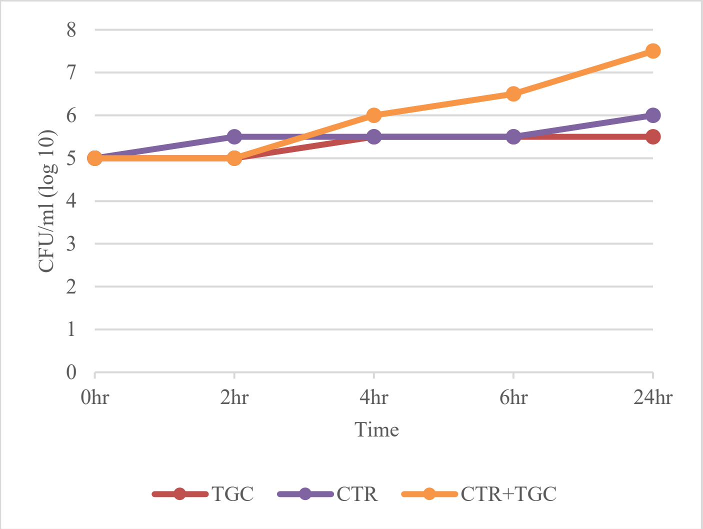

Figure 2(C) Time-Kill Assay (TKA) results represent the growth dynamics of *Escherichia coli* isolates treated with tigecycline (TGC), ceftriaxone (CTR), and the CTR + TGC combination. Bacterial growth (measured in CFU/ml, $\log_{10}$) was monitored over 24 hours. The CTR + TGC combination exhibited increased bacterial growth compared to the individual antibiotics, confirming an antagonistic interaction. Specifically, the bacterial count for the combination surpassed that of TGC and CTR after 4 hours, with sustained growth observed up to 24 hours.

Among the bacterial species tested, *Acinetobacter spp*. exhibited the highest number of synergistic interactions (22 isolates) and significant antagonistic effects (17 isolates), with comparatively fewer indifferent responses (9 isolates), highlighting its dynamic behavior toward antibiotic combinations. *E. coli* displayed a more balanced response, with synergy in 9 isolates, antagonism in 6 isolates, and indifference in 11 isolates. *Klebsiella spp*. predominantly exhibited indifferent effects (15 isolates), with limited synergy (11 isolates) and minimal antagonism (2 isolates), suggesting reduced responsiveness to the tested combinations. In contrast, *P. aeruginosa* showed equal numbers of synergistic and indifferent interactions (10 each), with no antagonistic responses (Figure 3), highlighting its distinct response profile among the tested species.

The DD method identified synergistic interactions in 58 isolates, of which 55% (32 isolates) were confirmed by TKA (Figure 4), demonstrating moderate concordance between the two methods. Similarly, among the 25 isolates displaying antagonistic effects by DD, 56% (14 isolates) were validated through TKA (Figure 4). These findings underscore the reliability of the DD method as a preliminary screening tool for detecting antibiotic interactions.

Also, PB + MRP emerged as the most frequently observed synergistic pair, identified in 19% of the isolates (Figure 5). This was followed by AK + CTR, detected in 16% of isolates, and MRP + TGC, observed in 13% of isolates. Other prominent combinations included MRP + AK (10%), CTR + TGC (8%), and MRP + AZM (8%). CTR paired with C was seen in 7% of the isolates, while PB + CTR was identified in 5% of the isolates. Less frequent combinations included CTR + COT (1%), CTR + PB (4%), MRP + COT (1%), and several others, showcasing a wide range of synergistic interactions across the isolates.

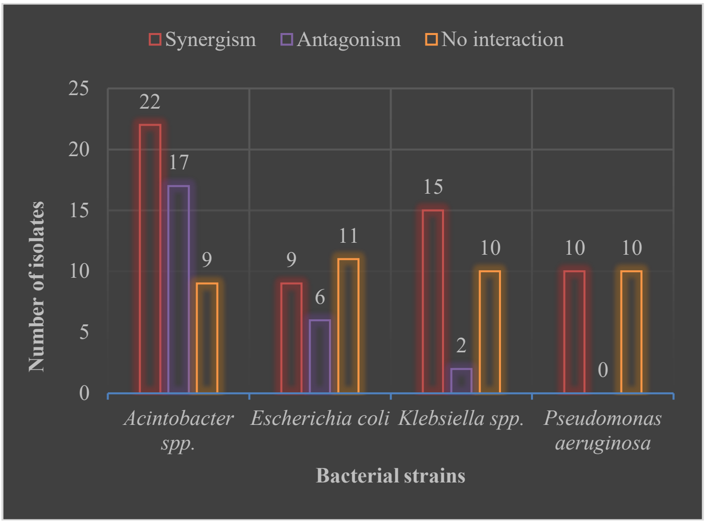

Figure 3 Organism-specific interactions observed in multidrug-resistant (MDR) bacterial isolates. A bar graph showing the distribution of synergistic, antagonistic, and no interactions across different bacterial species.

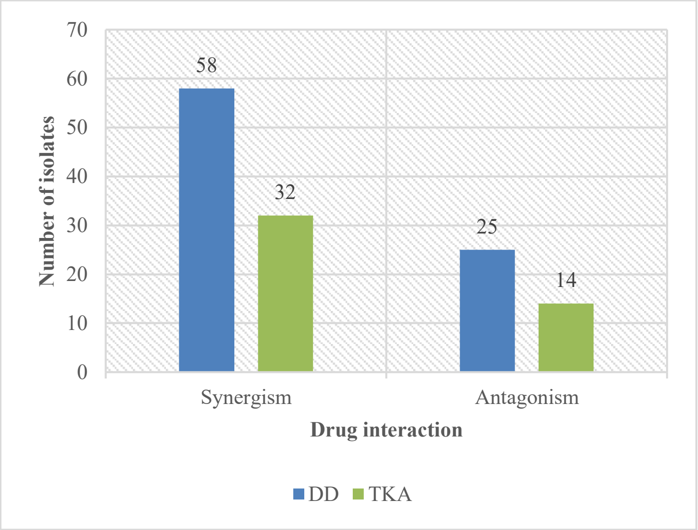

Figure 4 Comparison of synergism and antagonism as detected by the Disc Diffusion (DD) method and Time-Kill Assay (TKA). A bar graph showing the number of isolates with synergistic and antagonistic interactions.

For antagonistic interactions, CTR + TGC demonstrated the highest frequency, accounting for 40% of cases, followed by CTR + PB, which contributed to 24% of antagonistic interactions. The combination of CTR + COT accounted for 12%, while the rest were distributed among other combinations. These findings highlight the variability in antagonistic effects across different antibiotic pairs and underscore the importance of careful selection of antibiotic combinations for practical applications.

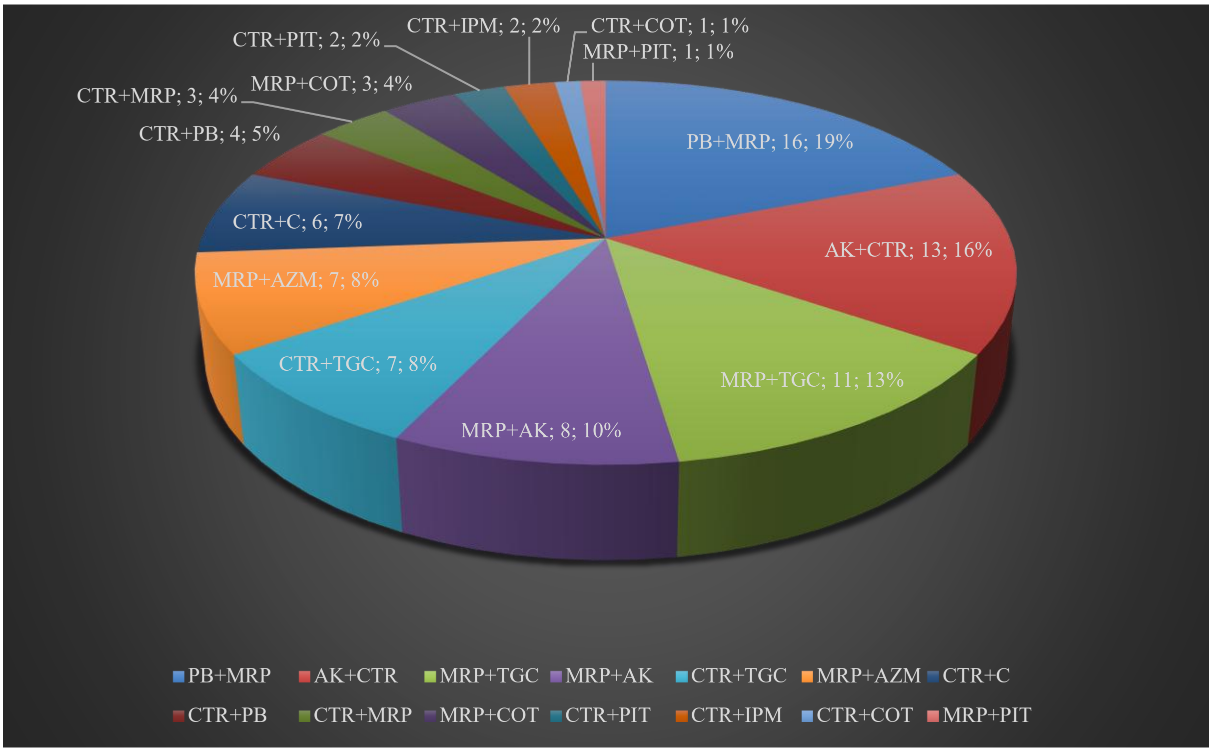

Figure 5 Distribution of synergistic interactions among antibiotic combinations. A pie chart representing the frequency of synergistic interactions for various antibiotic pairs. PB + MRP was the most frequently observed synergistic combination (19%), followed by AK + CTR (16%), MRP + TGC (13%), and several other combinations.

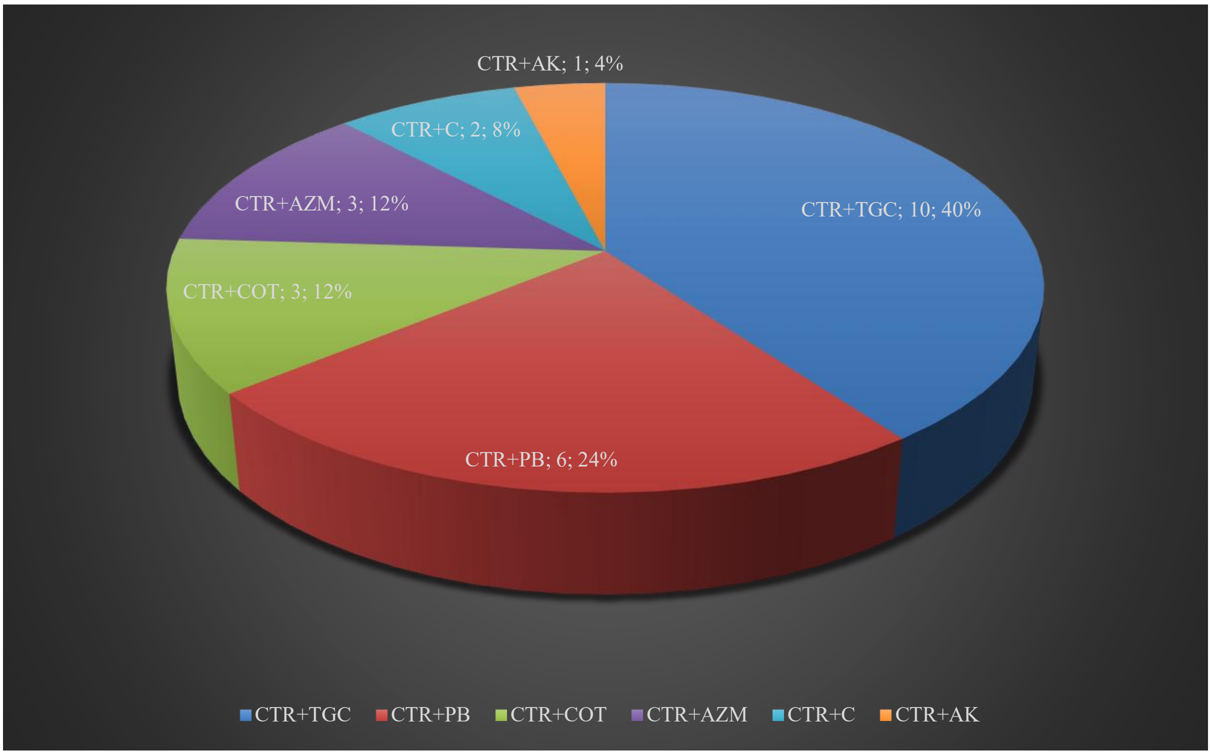

Figure 6 Distribution of antagonistic interactions among antibiotic combinations. A pie chart depicting the frequency of antagonistic interactions observed among tested antibiotic combinations. CTR+TGC showed the highest antagonism (40%), followed by CTR+PB (24%), CTR+COT (12%), and other combinations.

DISCUSSION

The present study highlights the critical interactions between antibiotic combinations against MDR bacterial strains, uncovering both synergistic and antagonistic effects with significant implications in practice. Notably, combinations such as CTR + TGC, PB + CTR, and CTR + COT demonstrated inconsistent results, showing synergism in some isolates and antagonism in others. This variability underscores a key challenge in MDR management – the necessity for individual and organism-specific testing. A single combination can produce contrasting effects depending on the bacterial strain, reinforcing the need for an individualized strategy for remedy.

However, current organism-specific testing methods are time-intensive and of a complex nature, thus posing a significant hurdle, particularly in high-stakes environments. Rapid identification of effective antibiotic combinations is vital for improving survival yet delays in delivering actionable results increase the risk of complications. This highlights the urgent need for faster, streamlined, and accessible antimicrobial susceptibility testing (AST) methods to ensure timely interventions and better outcomes.

Combination therapies leverage synergistic effects to enhance treatment outcomes, reduce dosage, and minimize toxicity ***(Laishram et al., 2017; Pietsch et al., 2021)***. Modifications to simple methods, like the disc diffusion (DD) technique, have long been explored to make susceptibility testing faster and more accessible ***(Chinwuba et al., 1991)***, particularly in the context of LMICs. The DD method has been reported to be very useful in determining the susceptibility and resistance to cefepime, particularly for patients admitted to the ICU with ventilator-associated pneumonia caused by *Acinetobacter* or *Klebsiella* (***Mahjobipoor et al., 2022***). Using early growth for DD testing as a simple and accurate tool for antibiotic susceptibility testing, ***Webbber et al., (2022)*** reported reduced time to results with no additional supply costs or equipment/instrumentation. More recently, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) has also recommended DD as a reproducible and accurate method for susceptibility testing of frequently isolated anaerobic bacteria (***Matuschek et al., 2023***).

Combination therapy is becoming increasingly critical in managing MDR infections, particularly in cases involving MDR or PDR strains of *P. aeruginosa* ***(Okoliegbe et al., 2021)***. Our findings reaffirm the importance of combination therapy, particularly in MDR infections. Combinations such as PB + MRP and AK + CTR emerged as the most frequent synergistic pairs in this study. Notably, these combinations are novel, as they have not been previously reported, providing new opportunities for combating resistant infections. Synergy with colistin-based combinations aligns with studies showing that colistin enhances bacterial membrane permeability, facilitating the efficacy of partner antibiotics ***(Tängdén et al., 2014)***. For instance, ***Laishram et al. (2017)*** reported synergy rates as high as 96.3% for colistin + MRP and 94.2% for colistin + rifampicin against *A. baumannii*. Similar effects were observed with combinations involving PB and MRP in *K. pneumoniae* ***(Laishram et al., 2017)***. Other studies have demonstrated comparable efficacy for colistin-based therapies combined with teicoplanin ***(Zafar et al., 2015)***.

In addition to polymyxin-based combinations, our study also identified AK + CTR as a synergistic pair, consistent with earlier reports of β-lactam–aminoglycoside combinations across multiple bacterial strains ***(Giamarellou et al., 1986; Chinwuba et al., 1991)***. Such synergistic effects are often influenced by MIC values, as observed in combinations involving sulbactam with MRP or doripenem, or doripenem with colistin, TGC, AK, or rifampicin ***(Laishram et al., 2017)***.

In contrast, antagonistic interactions were frequently observed with CTR + TGC, which showed the highest antagonism rates (40%) in the present study. This mirrors the findings in *K. pneumoniae* and other MDR strains, where suboptimal drug ratios and high MIC values led to antagonistic outcomes with combinations like colistin + ertapenem or IMP ***(Laishram et al., 2017)*** or levofloxacin–rifampin ***(Kang et al., 2023)***. These results suggest that inappropriate dosing and ratio optimization may exacerbate antagonistic effects, underscoring the importance of precise drug combinations to avoid failures of remedy.

While *in vitro* synergy offers promising insights, the literature indicates that these effects do not always translate into success ***(Laishram et al., 2017)***. This gap between laboratory findings and real-world outcomes highlights the need for further pre-clinical and clinical validation of synergistic combinations *in vivo* to ensure therapeutic efficacy.

As MDR infections continue to rise, advancements in AST technologies are essential to meet clinical demands. Emerging methods such as matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), flow cytometry, and isothermal microcalorimetry have shown potential for faster detection of antimicrobial resistance and effective combinations ***(Gajic et al., 2022)***. Integrating these tools into routine practice can significantly reduce turnaround times, facilitating timely treatment decisions for critically affected individuals. In parallel, computational approaches that combine *in silico* modeling with empirical laboratory validation can accelerate the discovery of synergistic antibiotic pairs ***(Zhu et al., 2021)***. High-resolution imaging and sequencing technologies will further refine our understanding of bacterial resistance mechanisms ***(Zhu et al., 2021)***, enabling the development of personalized strategies tailored to individual needs.

CONCLUSIONS

The present study emphasizes the importance of combination therapy in managing MDR infections, particularly through innovative synergistic combinations such as PB + MRP and AK + CTR. Using a single drug susceptibility testing (DST) plate allowed for the identification of at least six drug combinations, with results available within 24 hours. This methodology, which is less complex and more cost-effective than traditional approaches, supports the findings from TKA, thereby enhancing its credibility. Despite the limitations of this study including its small sample size and having one center, the findings suggest that DD method could serve as a valuable screening tool, particularly in critical care environments where rapid and effective antibiotic intervention is crucial for individuals affected with MDR pathogens. The ability to quickly and accurately identify synergistic or antagonistic drug interactions enables timely adjustments to antibiotic protocols, potentially improving outcomes in urgent situations. Overall, the advancement of this protocol represents a significant step forward in managing MDR infections, providing healthcare professionals with a practical and accessible resource to address the challenges of antibiotic resistance, particularly in middle- and low-income countries. However, further research is needed to expand the range of bacterial species for testing and investigate the role of additional antibiotics, thereby enhancing the screening process and validating the findings of the present study. Moreover, integrating molecular techniques with the proposed assay could offer valuable insights into the underlying mechanisms of MDR, facilitate a more targeted approach for remedy, and improve outcomes.

ABBREVIATIONS

AK: amikacin
AT: aztreonam
AMR: antimicrobial resistance
AST: antimicrobial susceptibility testing
AT: aztreonam
AZM: azithromycin
BALF: bronchoalveolar lavage fluid
C: chloramphenicol
CFU: colony forming units
COT: cotrimoxazole
COVID-19: coronavirus disease-2019
CPZ: cefoperazone
CRAB: carbapenem-resistant *A. baumannii-calcoaceticus*
CTR: ceftriaxone
DD: disc diffusion
DST: drug susceptibility testing
EUCAST: European Committee on Antimicrobial Susceptibility Testing
ICU: intensive care unit
IPM: imipenem
IZD: inhibition zone diameter
LE: levofloxacin
LOM: lomefloxacin
MDR: multidrug-resistant/multidrug resistance
MRP: meropenem
MHA: Mueller-Hinton Agar
MIC: minimum inhibitory concentration
MRP: meropenem
MRSA: methicillin-resistant *S. aureus*
PB: polymyxin B
TGC: tigecycline
TKA: time-kill assay
WHO: World Health Organization

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