

Evaluation of a ceftazidime-avibactam and aztreonam disc diffusion synergy test using the broth disc elution method as the reference standard

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ABSTRACT

Background: Ceftazidime-avibactam (CZA) combined with aztreonam (ATM) restores activity against metallo- β -lactamase-producing Enterobacterales. Broth disc elution (BDE) is recommended for testing this combination but is often impractical in resource-limited settings. This study determined the diagnostic accuracy of a CZA–ATM disc diffusion synergy test using BDE as the reference standard.

Material and Methods: This prospective cross-sectional study was conducted at the Armed Forces Institute of Pathology, Rawalpindi, from November 17, 2025, to May 17, 2026. A total of 140 non-duplicate carbapenem-resistant Enterobacterales isolates non-susceptible to CZA and ATM were tested in parallel by disc diffusion synergy and BDE using independent, blinded readers. Sensitivity, specificity, predictive values, accuracy, and Cohen's κ with 95% confidence intervals (CIs) were calculated.

Results: BDE detected synergy in 80 of 140 isolates (57.1%). Disc diffusion yielded 55 true positives, 60 true negatives, 25 false negatives, and no false positives. This demonstrated a sensitivity of 68.8% (95% CI 57.9–77.9), specificity of 100% (95% CI 94.0–100), positive predictive value of 100% (95% CI 93.5–100), negative predictive value of 70.6% (95% CI 60.2–79.2), accuracy of 82.1% (95% CI 75.0–87.6), and Cohen's κ of 0.65 (95% CI 0.54–0.77).

Conclusion: The disc diffusion synergy test showed 100% specificity but moderate sensitivity for in-vitro CZA–ATM synergy. A positive result reliably identifies synergy, whereas approximately one-third of synergistic isolates were missed.

Keywords: Antimicrobial susceptibility testing, Aztreonam, Broth disc elution, Carbapenem-resistant Enterobacterales, Ceftazidime-avibactam, Disc diffusion, Metallo- β -lactamase.

BACKGROUND

Carbapenem-resistant Enterobacterales (CRE) are highly concerning pathogens due to their extensive resistance profiles and restricted therapeutic options.¹ Antimicrobial resistance constitutes a significant global public health threat. The World Health Organization has classified CRE as Priority 1 (Critical) pathogens.² In serious infections, such as bloodstream and intra-abdominal infections, those caused by CRE are associated with prolonged hospitalizations, elevated

medical costs, and increased mortality rates.³ The synthesis of carbapenemases, classified by the Ambler system into Class A serine carbapenemases (e.g., *Klebsiella pneumoniae* carbapenemase [KPC]), Class B metallo- β -lactamases (MBLs; e.g., New Delhi metallo- β -lactamase [NDM], Verona integron-encoded metallo- β -lactamase [VIM], and imipenemase [IMP]), and Class D oxacillinases (e.g., OXA-48-like enzymes), constitutes the primary mechanism of carbapenem resistance in Enterobacterales.¹ The specific carbapenemase produced significantly influences the efficacy of newer β -lactam/ β -lactamase inhibitor combinations. Avibactam is ineffective against MBLs but it is effective against Class A and Class D carbapenemases when combined with ceftazidime (ceftazidime-avibactam, CZA).⁴ Conversely, co-produced serine β -lactamases such as extended-spectrum β -lactamases (ESBLs), AmpC enzymes, KPC, and OXA-48-like enzymes readily inactivate aztreonam (ATM), which is inherently resistant to hydrolysis by MBLs. Consequently, MBL-producing CRE isolates exhibiting multiple β -lactamase pathways are often

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resistant to aztreonam alone. The combination of CZA and ATM constitutes a rational therapeutic approach for these difficult infections, as avibactam protects aztreonam from hydrolysis by serine β -lactamases.⁵ The increasing clinical evidence demonstrating favourable outcomes with CZA–ATM therapy for infections caused by MBL-producing CRE underscores the need for reliable scientific methods to predict in-vitro synergy and guide suitable treatment.⁶ CRE, predominantly carbapenemase-producing strains, pose an increasing public health threat in Pakistan, where significant community carriage has been observed, with a reported prevalence of 14.4%.⁷ Consequently, in regions with a high prevalence of MBL-producing bacteria, accurate laboratory identification of the synergy between these medications is especially vital.

The predominant approach for evaluating CZA–ATM synergy is the broth disc elution (BDE) technique, supported by Clinical and Laboratory Standards Institute (CLSI) M100 (36th ed., 2026) recommendations.^{8,9} Nonetheless, its routine use in resource-limited settings is hindered by laborious preparation, meticulous handling and substantial resource requirements. Despite the introduction of many agar-based methods as simpler alternatives, their efficacy has been variable.¹⁰ This study therefore evaluated the diagnostic accuracy of a CZA–ATM disc diffusion synergy test against BDE as the reference method in clinical CRE isolates. The study assessed phenotypic CZA–ATM synergy in a selected population of CRE isolates; carbapenemase genes were not characterised, and the study therefore does not establish that the isolates tested were MBL producers.

MATERIAL AND METHODS

This prospective cross-sectional diagnostic accuracy study was conducted at the Department of Microbiology, Armed Forces Institute of Pathology (National University of Medical Sciences), Rawalpindi, Pakistan, from 17th November 2025 to 17th May 2026. The sample size was calculated with Buderer's formula¹¹ using an anticipated sensitivity of 70% for the disc diffusion synergy test, an absolute precision of $\pm 10\%$ and a 95% confidence level, which required at least 81 synergistic (BDE-positive) isolates. The prevalence of the target condition (BDE-confirmed CZA–ATM synergy) among CRE isolates non-susceptible to both agents was taken as approximately 58%, rather than the

14.4% community carriage prevalence of carbapenemase-producing Enterobacterales,⁷ giving a minimum required sample size of 140 isolates. Non-probability consecutive sampling was used: all non-duplicate CRE isolates recovered from clinical specimens during the study period were screened for eligibility, and 140 eligible isolates were enrolled.

Isolates were classified as CRE when they were non-susceptible (intermediate or resistant) to at least one carbapenem (imipenem, meropenem or ertapenem) by modified Kirby–Bauer disc diffusion, interpreted against the CLSI M100 (36th ed., 2026) breakpoints for Enterobacterales.⁸ Non-duplicate clinical CRE isolates non-susceptible to both CZA and ATM were included, because the CZA–ATM combination is considered in clinical practice only when neither agent is active alone. Isolates susceptible to either agent, repeat isolates from the same patient and contaminated cultures were excluded.

The study was approved by the Institutional Review Board (IRB) before its commencement, vide reference number FC-MIC-04/READ-IRB/24/3681 dated 18th December 2024.

Every isolate was tested in parallel by the index test (CZA–ATM disc diffusion synergy) and by the reference standard (BDE). The two tests were set up and read by different microbiologists, each without access to the other test's result; results were recorded on separate coded proformas bearing only the isolate laboratory number and were merged only after all testing had been completed. Bacterial isolates were identified to species level with the API 20E system (bioMérieux, Marcy-l'Étoile, France), and antimicrobial susceptibility was determined by modified Kirby–Bauer disc diffusion as described above.

BDE was performed according to CLSI M100 (36th ed., 2026)^{8,9} using cation-adjusted Mueller–Hinton broth. ATM (30 μ g) and CZA (30/20 μ g) discs (Oxoid™) produced final concentrations of 6 μ g/mL ATM, 6 μ g/mL ceftazidime and 4 μ g/mL avibactam. A 0.5 McFarland suspension was prepared from 18–24-hour growth. Four tubes (ATM alone, CZA alone, the ATM+CZA combination and a growth control) were prepared for each isolate. After 30 minutes of disc elution, 25 μ L of inoculum was added to achieve approximately 7.5×10^5 colony-forming units (CFU)/mL. Tubes were incubated at 33–35 °C for 16–20 hours. Synergy was recorded only when the growth

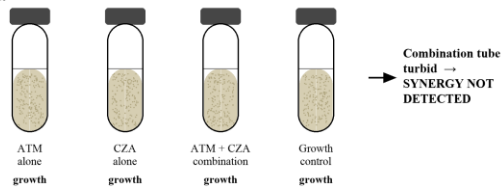
control and both single-agent tubes showed visible growth (turbidity or a definite button of growth) while the ATM+CZA combination tube showed no visible growth; visible growth in the combination tube was recorded as no synergy (Figure-I). Purity was confirmed on blood agar; tube sets with no growth in the growth control or with a mixed culture were regarded as invalid and repeated from a fresh subculture.



Figure I (a)

Broth disc elution (BDE): four-tube set-up and interpretation

A. Synergy not detected



B. Synergy detected

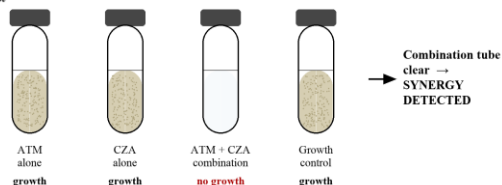


Figure I (b)

Figure-I: Broth disc elution (BDE) reference test. (a) Photograph of two BDE tubes from the present study. (b) Schematic of the complete four-tube set-up used for every isolate: aztreonam (ATM) alone, ceftazidime-avibactam (CZA) alone, the ATM+CZA combination and a growth control.

Synergy was recorded only when the growth control and both single-agent tubes showed visible growth (turbidity) while the ATM+CZA combination tube remained clear. In panel (a) the tube labelled "2" shows visible growth in the ATM+CZA combination tube and was read as synergy not detected, whereas the tube labelled "3" shows a clear ATM+CZA combination tube and was read as synergy detected.

The disc diffusion synergy test was performed on Mueller–Hinton agar using a 0.5 McFarland suspension. CZA (30/20 µg) and ATM (30 µg) discs (Oxoid™) were

placed 20 mm apart, centre-to-centre, and incubated at $35 \pm 2^\circ\text{C}$ for 16–20 hours. The plate was read against a dark background with transmitted light, and the zone edge was taken as the point of complete inhibition of growth. Using the criteria described by Verschelden *et al.*,¹² synergy was recorded when any one of the following was present in the area between the two discs: (i) enhancement, the appearance of a zone of inhibition around the ATM disc in an isolate that showed no ATM zone when ATM was tested alone; (ii) distortion, a visible extension of the ATM inhibition zone on the aspect facing the CZA disc compared with the aspect facing away from it, so that the zone was no longer circular; or (iii) bridging, confluence of the ATM and CZA inhibition zones (Figure-II). Plates showing none of these three features were recorded as no synergy. Every plate was read independently by two microbiologists who were blinded to the BDE result; the few discordant readings were resolved by consensus. Interobserver agreement was not formally quantified.



Figure II (a)



Figure II (b)

Figure-II: Disc diffusion synergy patterns between the aztreonam (ATM) and ceftazidime-avibactam (CZA) discs. (a) Enhancement (arrow). (b) Distortion of the ATM inhibition zone towards the CZA disc (arrow), so that the zone is no longer circular. Both patterns, as defined in the text, were read as synergy detected.

Quality control was performed on each testing day using *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC BAA-1705. *E. coli* ATCC 25922 was used to verify the performance of the ATM and CZA discs, the broth and the inoculum, and its zone diameters were required to fall within the CLSI M100 acceptable ranges. *K. pneumoniae* ATCC BAA-1705 is

a KPC-producing strain whose carbapenemase is inhibited by avibactam; it was therefore included as a synergy-negative control, being expected to grow in the ATM tube, to show no growth in the CZA tube, and to show no enhancement, distortion or bridging in the disc diffusion synergy test. A run was accepted only when both control strains gave the expected results; otherwise, the whole run was repeated and its clinical isolate results were discarded. No MBL-producing, synergy-positive reference strain was available locally.

Data were analysed using SPSS version 27. Categorical variables were summarised as frequencies and percentages. Using BDE as the reference standard, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and overall accuracy were calculated with 95% confidence intervals (CIs) derived by the Wilson score method. Agreement beyond chance between the two methods was assessed with Cohen's κ , reported with its 95% CI (asymptotic standard-error method) and interpreted according to the Landis and Koch criteria. Because both tests yield a binary (synergy/no synergy) result, rank correlation and receiver-operating-characteristic analysis add no information beyond the 2×2 table and were not performed; no formal hypothesis test was applied, and results are presented as point estimates with 95% CIs. All 140 enrolled isolates gave valid, interpretable results

by both tests and were included in the analysis; no isolate was excluded because of an invalid, contaminated or indeterminate result.

RESULTS

Among the 140 isolates, *Klebsiella pneumoniae* (55.7%) and *Escherichia coli* (29.3%) predominated. Urine was the most frequent specimen (32.1%), and 57.9% of isolates originated from intensive care units (Table-I).

Using BDE as the reference comparator, 80 isolates (57.1%) were positive and 60 (42.9%) were negative for synergy. The disc diffusion synergy test identified 55 true positives and 60 true negatives, with 25 false-negative and no false-positive results (Table-II). Sensitivity was 68.75% (95% CI 57.93–77.85) and specificity 100% (95% CI 93.98–100); the predictive values, overall accuracy and false-negative rate are given in Table-III. The positive likelihood ratio could not be estimated because no false-positive result occurred, and the negative likelihood ratio was 0.31.

Agreement between the disc diffusion synergy test and BDE was substantial, with a Cohen's κ of 0.65 (95% CI 0.54–0.77).

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Table-I: Clinical and Microbiological Characteristics of the 140 Study Isolates.

Characteristic	Category	No. of isolates	Percentage (%)
Species distribution	<i>Klebsiella pneumoniae</i>	78	55.7
	<i>Escherichia coli</i>	41	29.3
	<i>Enterobacter cloacae</i> complex	12	8.6
	<i>Citrobacter freundii</i>	5	3.6
	<i>Proteus mirabilis</i>	3	2.1
	<i>Klebsiella oxytoca</i>	1	0.7
Clinical specimen source	Urine	45	32.1
	Blood	35	25.0
	Respiratory specimens (sputum, BAL, TA)	27	19.3
	Pus/wound swab	20	14.3
	Tissue/device-related specimens	13	9.3
Ward of origin	Medical ICU (MICU)	35	25.0
	Surgical ICU (SICU)	28	20.0
	General medical ward	24	17.1
	General surgical ward	18	12.9
	Paediatric ICU (PICU)	12	8.6
	Neonatal ICU (NICU)	6	4.3
	Other/outpatient	17	12.1
	Urinary tract infection	45	32.1
Clinical category	Bloodstream infection/sepsis	35	25.0
	Lower respiratory tract infection	27	19.3
	Skin and soft tissue infection	20	14.3
	Healthcare-associated device-related infection	13	9.3

Abbreviations: BAL, bronchoalveolar lavage; TA, tracheal aspirate; ICU, intensive care unit.

Table-II: Cross-classification of the disc diffusion synergy test against broth disc elution.

Reference Standard (BDE)	Disc Diffusion Positive	Disc Diffusion Negative	Total
Positive	55 (TP)	25 (FN)	80
Negative	0 (FP)	60 (TN)	60
Total	55	85	140

TP, true positive; FN, false negative; FP, false positive; TN, true negative; BDE, broth disc elution.

Table-III: Diagnostic performance of the CZA–ATM disc diffusion synergy test against broth disc elution (n = 140).

Diagnostic measure	Estimate	95% CI
Sensitivity	68.75%	57.93–77.85
Specificity	100%	93.98–100
Positive predictive value	100%	93.47–100
Negative predictive value	70.59%	60.18–79.21
Overall accuracy	82.14%	74.97–87.60
False-negative rate	31.25%	22.15–42.07
Cohen's κ	0.65	0.54–0.77
Negative likelihood ratio	0.31	Not estimable*
Positive likelihood ratio	Not estimable*	–

DISCUSSION

In this single-centre diagnostic accuracy study, a disc diffusion-based CZA plus ATM synergy assay was evaluated against BDE as the reference method for detecting in-vitro synergy among CRE. The assay demonstrated 100% observed specificity, indicating that all isolates classified as synergistic by disc diffusion were also synergistic by BDE. However, sensitivity was 68.75%, with approximately one-third of synergistic isolates missed by the index method. Thus, a positive disc diffusion result reliably identified in-vitro synergy, whereas a negative result did not exclude it.

Phenotypic diffusion-based approaches, including disc approximation, gradient diffusion and double-disc synergy methods, have generally demonstrated high specificity but variable sensitivity when compared with reference methods such as broth microdilution or BDE.¹³ Reduced sensitivity of diffusion-based synergy assays may be particularly relevant in isolates with complex β -lactamase profiles, including co-production of multiple carbapenemases, ESBLs and AmpC enzymes, where the incremental increase in inhibition-zone diameter may be small and difficult to distinguish reproducibly.¹⁴ In contrast, broth-based methods provide a more homogeneous and controlled antimicrobial environment, allowing a more consistent interaction between the agents and the bacterial inoculum and consequently greater agreement with reference susceptibility methods.^{15,16} However, these advantages are accompanied by greater methodological complexity and resource requirements, which may limit

their routine implementation, particularly in laboratories with constrained resources.¹⁷ The specific cause of the 25 false-negative results in the present study cannot be established. One possible explanation is an intrinsic limitation of diffusion-based testing, whereby the concentration and spatial distribution of avibactam reaching the region between the discs may be insufficient to adequately inhibit co-produced serine β -lactamases and thereby reveal the activity of aztreonam. Technical and observer-related factors may also contribute. These possibilities cannot be distinguished without repeat testing and molecular characterisation; the false-negative rate should therefore be regarded as a limitation of the assay format rather than attributed to laboratory error.

The clinical and laboratory relevance of CZA–ATM synergy testing is particularly important in regions where MBL-producing Enterobacterales are prevalent. NDM-producing organisms frequently co-harbour ESBL, AmpC or OXA-48-like enzymes, creating a phenotype in which aztreonam alone may be compromised by co-produced serine β -lactamases, while avibactam can inhibit these enzymes and thereby restore aztreonam activity.^{18,19} Regional surveillance from Pakistan and neighbouring countries has documented a substantial burden of CRE, with NDM among the important carbapenemase determinants.²⁰ In such settings, a simple phenotypic synergy assay may provide a practical approach for identifying potentially active CZA–ATM combinations where molecular

diagnostics or advanced susceptibility-testing platforms are unavailable.

KPC and OXA-48-like carbapenemases are prominent in Europe, the United Kingdom and North America^{21, 22}, whereas NDM and other MBLs are widely distributed across Asia, the Middle East, Africa and parts of Latin America.²³ In KPC-producing organisms, CZA generally retains intrinsic activity, reducing the need to depend on CZA-ATM combination therapy. In contrast, the increasing global dissemination of MBL-producing *Enterobacterales*^{20,23} and the emergence of isolates carrying multiple carbapenemase genes¹⁹ have increased the clinical demand for robust methods to identify effective β -lactam combinations.²⁴

The diagnostic asymmetry observed in this study — 100% observed specificity with imperfect sensitivity — has important implications for laboratory interpretation. This pattern is consistent with the previously reported performance of diffusion-based synergy assays²⁵, which tend to have a high positive predictive value and a lower negative predictive value.¹² Negative results should therefore be interpreted with caution, particularly in critically ill patients with bloodstream infection, ventilator-associated pneumonia or other severe CRE-related syndromes in which there is little room for therapeutic error. The assay is inexpensive and can be incorporated into an existing disc diffusion workflow, and its moderate sensitivity supports a tiered approach in which a positive result is accepted as a rule-in finding and negative results are confirmed by BDE, particularly in resource-limited laboratories.¹⁷ The prospective design, the mutually blinded reading of the index test and the reference standard, the inclusion of clinically relevant CRE isolates and the use of a broth-based reference standard strengthen the internal validity of this study.

Several limitations should also be acknowledged. The study was conducted at a single centre with a moderate sample size, which limits generalisability. Only isolates non-susceptible to both CZA and ATM were studied, so the estimates of sensitivity, specificity, PPV and NPV apply to that deliberately selected population, in which the prevalence of synergy was 57.1%, and not to unselected CRE isolates; because PPV and NPV depend on prevalence, they should not be applied to settings with a different proportion of synergistic isolates. Molecular characterisation was not performed; the isolates were therefore not confirmed to be MBL

producers, and carbapenemase genotype could not be correlated with phenotypic synergy. Species identification used a biochemical identification system rather than mass spectrometry or molecular methods, which may limit precision for some closely related *Enterobacterales*. No MBL-producing, synergy-positive reference strain was available for quality control. Interobserver agreement was not formally quantified, and clinical outcomes were not assessed. Future multicentre studies should include larger and more diverse isolate collections, molecular characterisation, standardised interpretive criteria and clinical outcome data to define the diagnostic and therapeutic relevance of CZA-ATM synergy testing more precisely.

CONCLUSION

Compared with BDE, the CZA-ATM disc diffusion assay showed 100% observed specificity but only moderate sensitivity for the detection of in-vitro phenotypic synergy, and approximately one-third of synergistic isolates were missed. A positive result reliably indicated in-vitro synergy in this study, whereas a negative result did not exclude synergy. These findings relate to in-vitro phenotypic synergy testing only and do not, by themselves, establish the clinical effectiveness of the CZA-ATM combination.

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DISCLOSURES

None

CONFLICT OF INTEREST

None

GRANT SUPPORT & FINANCIAL DISCLOSURE

Declared none

AUTHOR CONTRIBUTION

Faiza Zaheer: Contributed to substantially conception and study design, data acquisition, interpretation, critical revisions, final approval, accountable for all aspects of publication

Faisal Hanif: Contributed to analysis and interpretations, critical review, revision for important intellectual, final approval, accountable for all aspects of publication

Takeenah Hussain Naqvi: Contribution to study methodological and interpretation of microbiological findings, data analysis and interrelation, final approval, accountable for all aspects of publication

Anam Imtiaz: Contributed to data curation, critical review, final approval, accountable for all aspects of publication

Farooq Ahmad: Contributed to acquisition of clinical and microbiological data, lab investigation, data verification and interpretation of results, critical review, final approval, accountable for all aspects of publication

Muhammad Shabbir: Data analysis, interpretation of study findings, manuscript drafting, critical revisions, final approval, accountable for all aspects of publication

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