e-ISSN: 0976-822X, p-ISSN:2961-6042

Available online on http://www.ijcpr.com/

International Journal of Current Pharmaceutical Review and Research 2026; 18(6); 1429-1435

Original Research Article

Assessment of the Colistin Broth Disk Elution Method Against Reference Microbroth Dilution for MDR Gram-Negative Pathogens in a Low-Resource Setting

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Received: 01-04-2026 / Revised: 25-05-2026 / Accepted: 12-06-2026
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Conflict of interest: Nil

Abstract

Objective: To evaluate the performance of the Colistin Broth Disk Elution (CBDE) method for colistin susceptibility testing, compared with the reference broth microdilution (rBMD) method, among multidrug-resistant (MDR) Gram-negative bacteria.

Materials & Methods: This prospective study was conducted in a microbiology department of a multispecialty hospital and included 100 MDR isolates: 25 each of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter calcoaceticus-baumannii* complex. All isolates were tested for colistin susceptibility using both CBDE and rBMD methods as per standard guidelines.

Statistical Analysis: Agreement analysis between CBDE and rBMD was performed using categorical agreement (CA) and essential agreement (EA). Error rates were calculated and classified as major errors (ME) and very major errors (VME).

Results & Conclusions: CBDE demonstrated ≥90% categorical and essential agreement with rBMD across all isolates. Among Enterobacterales, agreement was 88% (22/25) for *E. coli* and 92% (23/25) for *K. pneumoniae*. For non-fermenters, agreement was 96% (24/25) for *P. aeruginosa* and 92% (23/25) for *Acinetobacter* complex. A total of eight errors (8%) were observed, including six major errors (6%) and two very major errors (2%). CBDE showed high concordance with rBMD, supporting its use as a reliable and cost-effective alternative for colistin susceptibility testing, particularly in resource-limited settings. However, larger multicentric studies are recommended for further validation.

Limitations: The relatively small sample size may have influenced the observed error rate. Larger multicentric studies are warranted to validate these findings further.

Keywords: Colistin Broth Disk Elution, Enterobacterales, Non-fermenter, reference Broth Microdilution.
DOI: 10.25258/ijcpr.18.6.231

Introduction

Infections caused by multidrug-resistant (MDR) gram-negative organisms, particularly carbapenem-resistant Enterobacterales (CRE) and non-fermenters, represent an escalating challenge in healthcare environments globally. These infections are challenging to manage due to the restricted therapeutic options available. In response to the increasing prevalence of antimicrobial resistance, particularly against second-line antimicrobial agents, the utilization of polymyxin drugs, specifically colistin and polymyxin B, has experienced a resurgence over the past decade. These drugs are now employed as a last resort for salvage therapy against infections caused by multidrug-resistant (MDR) organisms.[1]The extensive use of colistin has resulted in the global emergence of colistin-resistant strains. The Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) have endorsed the reference broth microdilution method (rBMD) as the standard procedure for assessing colistin susceptibility.[2,3,4]While the reference broth microdilution (rBMD) method is a precise technique for determining the minimum inhibitory concentration (MIC) of colistin, it may pose significant resource demands on clinical microbiology laboratories in developing countries.[5] While disk and gradient diffusion methods offer a more straightforward approach to

assessing colistin susceptibility, they are not endorsed by either the CLSI or the EUCAST due to their unacceptably high error rates. Consequently, laboratories are left without a practical method for determining colistin susceptibility.[6] The CLSI has recently introduced the Colistin Broth Disc Elution (CBDE) method as a means for evaluating colistin susceptibility. This method is both promising and straightforward, offering a viable alternative to the gold-standard rBMD method for colistin susceptibility testing.[3] Research has shown that the CBDE method achieves an essential and categorical agreement rate of over 95% to 100% when compared to the rBMD method for Enterobacterales, *Pseudomonas*, and *Acinetobacter* species.[7] This study aimed to evaluate the accuracy of the user-friendly CBDE method in comparison to the rBMD method for determining colistin susceptibility.

Materials and Methods

In the present study, CBDE was assessed in comparison to a commercially available kit (HI Media, Mumbai, India) utilizing the CLSI standards (M23, 5th edition, and M07, 11th edition) for the validation of alternative reference susceptibility tests.[8,9]

Bacterial isolates: A total of 100 non-repetitive MDR Gram-negative isolates were included in the study, consisting of 25 *Escherichia coli*, 25 *Klebsiella pneumoniae*, 25 *Pseudomonas aeruginosa*, and 25 *Acinetobacter cbc*. These isolates were derived from various clinical samples, including blood and body fluids, collected at our hospital between January 2022 and January 2023. The isolates were subsequently assessed for colistin susceptibility and were preserved at -70°C until the completion of processing.

Reference Broth Microdilution Method (rBMD): Susceptibility to colistin, within a test MIC range of 32–0.125 µg/mL, was assessed using a commercially available broth microdilution method in cation-adjusted Mueller-Hinton broth (CA-MHB-BD) on microtiter plates (HI Media, Mumbai, India). The results were interpreted according to the breakpoints specified in CLSI M100-2023.[10] Isolates with a colistin MIC of ≤2 µg/mL were classified as susceptible, whereas those with MICs of ≥4 µg/mL, specifically 8 µg/mL, 16 µg/mL, and 32 µg/mL, were deemed resistant for both Enterobacterales and non-fermenters, including *Pseudomonas aeruginosa* and *Acinetobacter cbc.*[10] The broth microdilution method was employed as the reference method, as recommended by the CLSI.[8]

Colistin broth disk elution (CBDE) tube test: To conduct the CBDE test, colistin concentrations of 0, 1, 2, and 4 µg/ml were established by incorporating zero disks (tube 0; serving as the growth control), one disk (tube 1), two disks (tube 2), and four disks (tube 3), respectively. The colistin was permitted to elute from the disks at ambient temperature for a duration of 60 minutes. The bacterial inoculum was prepared by suspending fresh colonies in normal saline and adjusting the turbidity to match the 0.5 McFarland standard (~10^8 CFU/mL). Subsequently, a volume of 50 µL of the bacterial inoculum was introduced into each tube containing colistin or the growth control. The tubes were securely sealed, and each inoculated tube was gently vortexed at a low speed. This slow speed is recommended to prevent colistin from sticking to the cap and the glass surface above the liquid's meniscus. It is advised to slightly loosen the caps before incubation. Tubes and purity plates were incubated for 16–20 hours for Enterobacterales *and Pseudomonas aeruginosa*, and for 20-24 hours for *Acinetobacter cbc* at 37°C before reading. A 10-µL loop was employed to transfer a subculture from the original inoculum tube to a blood agar plate for a purity check.[8]

Testing strategy: The CBDE and rBMD tests were conducted simultaneously on both retrospective and prospective isolates that had been preserved at -70°C. The procedure was repeated for certain strains when an inconsistent growth pattern was detected, such as no growth at 2µg/mL but growth at 1 µg/mL and 4µg/mL.

This irregular growth pattern could have been caused by contamination at higher dilutions, hetero-resistance, or incorrect concentrations of antimicrobial agents in the tubes.

Quality Control: For quality control purposes, the reference bacterial strains utilized were as follows: *E. coli* National Culture Type Collection (NCTC)13846, which is an mcr-1-positive strain; the susceptible *E. coli* strain, American Type Culture Collection (ATCC) 25922, which is mcr-1-negative; and the susceptible *P. aeruginosa* strain ATCC 27853.

Reporting: The purity plates were assessed to confirm the purity of the inoculum, specifically the growth control tube, which must exhibit clear turbidity for the test to be considered valid. The MIC was determined as the lowest concentration that completely inhibited visible growth in the test tubes.

The MIC results were interpreted as "Intermediate susceptible" (I) and "Resistant" (R) according to the CLSI guidelines. The MIC interpretative criteria for Enterobacterales and non-fermenters are presented in Table 1.[10]For comparisons of the two methods used for colistin susceptibility, the following agreements were calculated. [4]

Table 1: Reference MIC values of colistin [9,10]

	Minimum Inhibitory Concentration (MIC)	
	I	R
Enterobacterales	≤ 2	≥ 4
Nonfermenters	≤ 2	≥ 4

Footnotes: I-Intermediate Susceptible, R- Resistant

Categorical agreement (CA) is calculated by comparing the results of two different diagnostic methods (such as a new test versus a reference method) to determine how often they classify samples into the same categories, such as "susceptible," "intermediate," or "resistant."

Formula for Categorical Agreement [11]

CA (%) = Number of matching results/Total number of results×100

Essential agreement (EA) is used to measure the consistency of quantitative results, such as MICs, between a test method and a reference method. The results are considered in "essential agreement" if they are within a predefined acceptable range (typically ±1 doubling dilution for MIC values).

Formula for Essential Agreement [11]: EA (%) =Number of results within ±1 dilution / Total number of results×100

Interpretation for CA and EA [11, 12]: CA ≥ 90% is generally considered acceptable as per the current standards for many tests, such as antimicrobial susceptibility testing. Any deviation from this standard may require further investigation or adjustment in the test methodology.

An EA≥90% is generally considered acceptable in many diagnostic testing contexts, such as antimicrobial susceptibility testing.

Calculation of Errors: Whenever the results of the two diagnostic tests do not match, the errors are calculated as per the Food and Drug Administration, Antimicrobial Susceptibility Test Interpretive Criteria (FDA-STIC), 2021.[13] Minor error (mE) is characterized by a discrepancy where the isolate is classified as susceptible or resistant (S/R) according to the rBMD method, while being categorized as intermediate susceptible (I) by the CBDE method, or vice versa. Major error (ME) is characterized by a scenario in which the isolate is classified as intermediate susceptible according to the rBMD method, yet is deemed resistant when assessed using the CBDE method. Very major error (VME) is characterized by a scenario in which the isolate is determined to be resistant according to the rBMD method, yet is classified as intermediately susceptible when assessed using the CBDE method.

Statistical analysis: We have done agreement analysis between the two tests and found that the CBDE method demonstrated high concordance with the reference broth microdilution method, achieving ≥90% categorical and essential agreement overall. The overall error rate was 8%, comprising 6% major errors and 2% very major errors, indicating acceptable diagnostic performance of CBDE with minor variability in species-specific agreement

Results

Percentage Resistance: In this study, one hundred MDR bacterial isolates, each exhibiting resistance to at least one antimicrobial agent from three or more antimicrobial classes, were selected. The sample comprised 25 isolates each of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter cbc*. Resistance to colistin was observed in 16% of *E. coli*, 20% of *K. pneumoniae*, 16% of *P. aeruginosa*, and 24% of *Acinetobacter cbc* strains, as determined by the reference broth microdilution method. The CBDE method was concurrently employed for all these isolates to enable a comparative analysis between the two methodologies.

The results were interpreted for colistin as falling within the intermediate susceptible (I) and resistant (R) categories, in accordance with CLSI guidelines.[9] (Table 2) To evaluate the accuracy of the novel CBDE susceptibility method in comparison to the standard rBMD methods, both categorical and essential agreements were assessed.

In the context of gram-negative isolates, susceptibility testing for colistin demonstrated over 90% CA and EA between the two methods assessed. Specifically, for Enterobacterales, CA was ≥90% and EA was ≥90% (45/50), although individual results were slightly lower, with *K. pneumoniae* at 88% (22/25) and *E. coli* at 92% (23/25) [Table 3].

Comparable outcomes were observed for non-fermenters, with both CA and EA reaching ≥90% (47/50) between the two methods. Individually, the methods exhibited >95% agreement for *P. aeruginosa* (24/25) and 92% agreement for *Acinetobacter cbc* (23/25) [Table 4]. Among the one hundred isolates tested, a total of eight errors (8%) were identified, of which six were major errors and two were very major errors.

Table 2: MICs of colistin for Enterobacterales and non-fermenters determined by the reference Broth Microdilution and CBDE methods.

Isolates	MIC (number of isolates) rBMD				MIC (number of isolates) CBDE				Errors
	≤1 µg/ml	2 µg/ml	4 µg/ml	≥4 µg/ml	≤1 µg/ml	2 µg/ml	4 µg/ml	≥4 µg/ml	
E. coli	16	5	2	2	16	5	1	3	1ME, 1 VME
Klebsiella pneumoniae	6	14	0	5	6	11	2	6	3 ME
Pseudomonas aeruginosa	9	12	1	3	9	11	2	3	1ME
Acinetobacter cbc	6	14	1	4	6	14	1	4	1ME, 1VME

Footnotes: rBMD- reference Broth microdilution, CBDE- Colistin Broth Disk Elution, ME- Major Error, VME- Very Major Error, MIC- Minimum Inhibitory Concentration.

Table 3: Colistin MIC (µg/mL) reference BMD V/s CBDE method for *Escherichia Coli* [A] and *Klebsiella pneumoniae*[B]

Reference broth microdilution (rBMD) MIC (µg/mL)

Colistin Broth Disk Elution MIC (µg/mL)

A	≤1	2	4	≥4
≤1	16			
2		4	1[b]	
4			1	
≥4		1[a]		2

B	≤1	2	4	≥4
≤1	6			
2		11		
4		2[a]		
≥4		1[a]		5

The MIC agreements found via both methods are highlighted in yellow.
a- Major Errors (ME) – green
b- Very major Errors (VME)- Pink

Table 4: Colistin MIC (µg/mL) reference BMD V/s CBDE method for nonfermenters, *Pseudomonas aeruginosa*[C], and *Acinetobacter cbc* D], respectively

Reference broth microdilution (rBMD) MIC (µg/mL)

Colistin Broth Disk Elution MIC (µg/mL)

C	≤1	2	4	≥4
≤1	9			
2		11		
4		1[a]	1	
≥4				3

D	≤1	2	4	≥4
≤1	6			
2		13	1[b]	
4		1[a]		
≥4				4

The MIC agreements found via both methods are highlighted in yellow.
a- Major Errors (ME) – green
b- Very major Errors (VME)- Pink

Discussion

Colistin, an antimicrobial agent with a history spanning five decades, has regained prominence in the 21st century as a response to the escalating threat posed by infections caused by carbapenem-resistant Enterobacteriaceae (CRE). Despite ongoing debates about the pharmacokinetics and pharmacodynamics of polymyxins, colistin use has increased due to the scarcity of alternative antimicrobial agents. This increased usage has consequently led to the emergence of colistin-resistant pathogens.[14] While disk diffusion and gradient diffusion methods are alternative approaches for susceptibility testing, they are not recommended for assessing polymyxin susceptibility. These methods frequently yield high rates of false-susceptibility results, thereby reducing their reliability for accurately evaluating polymyxin susceptibility. [5,6,15]

Given that colistin is frequently regarded as the last line of defence against multidrug-resistant (MDR) gram-negative organisms, it is imperative to routinely assess its minimum inhibitory concentration (MIC) to ensure precise susceptibility results for this critical antibiotic. The broth microdilution (BMD) method is the reference technique recommended by the Clinical and Laboratory Standards Institute (CLSI) for determining the MIC of polymyxins.[8] This method is labour-intensive, costly, and time-consuming, and the propensity of polymyxins to adhere to the plastic surface of polystyrene plates further complicates the process, thereby exacerbating the challenges of accurate testing.[8,16] Consequently, there is a significant need for a rapid and user-friendly colistin susceptibility test in diagnostic clinical microbiology laboratories.[14]

In 2019, the CLSI Antimicrobial Susceptibility Testing Subcommittee endorsed the CBDE and CAT methods for colistin testing in Enterobacterales and *P. aeruginosa* isolates.[7] In the present study, we assessed the CBDE method for determining colistin susceptibility in one hundred multidrug-resistant Gram-negative isolates, including Enterobacterales and non-fermenters. Overall, we achieved over 90% categorical agreement and between 88% to 97% essential agreement for CBDE compared to the reference BMD method. We identified a total of 8 errors: 6 out of 8 (6%) were major errors (MEs), where rBMD classified the isolates as intermediate susceptible (I) while CBDE labelled them as resistant (R), and 2 out of 8 (2%) were very major errors (VMEs), where rBMD indicated resistance (R) but CBDE categorized them as intermediate susceptible (I).

Koyuncu O.O. et al. [17] conducted a study comparing the BD Phoenix 100 system (Becton Dickinson, USA) and the CBDE method against BMD for evaluating the in vitro efficacy of colistin against gram-negative bacteria. The study reported a high categorical agreement of 99.3% between the CBDE method and rBMD, with minimal error rates (0.2% very major errors and 0.5% major errors). Conversely, the BD Phoenix 100 system demonstrated a 95% categorical agreement with rBMD but exhibited a significantly higher error rate of 5%.[17] These results indicate that the CBDE method is a reliable alternative to the rBMD

method, whereas the BD Phoenix 100 system may be less effective in distinguishing between colistin-resistant and colistin-susceptible strains due to its elevated error rate.

A multicentre study conducted by the Clinical and Laboratory Standards Institute's Antimicrobial Susceptibility Testing Subcommittee evaluated two methods, namely the CBDE test and the CAT, to ensure accurate colistin susceptibility testing.[7] The authors reported a 94.4% essential agreement (EA) and a 97.9% categorical agreement (CA) with reference broth microdilution MICs using the CBDE method.[7] The observed errors were minimal (VME-3.2% and ME-0.9%), as this multicentre study included a large number of isolates (Enterobacterales-270 isolates and nonfermenters-228) compared to our study.

Simner et al.[18] emphasized in their study that this test can address numerous challenges in determining colistin susceptibility and may serve as a practical, accurate, and reproducible alternative for laboratories of varying sizes. While numerous studies have assessed the efficacy of this test in detecting colistin resistance in Enterobacterales, there is a paucity of research evaluating its application for *P. aeruginosa* isolates. Humphries et al. [7] reported a categorical agreement of 99.3% for the CBDE method in *P. aeruginosa*, with no very major errors (VME) detected and a minor error (ME) rate of 0.7%. Based on these findings, they concluded that the CBDE method is appropriate for determining colistin susceptibility in *P. aeruginosa* isolates. Conversely, they advised against using the CBDE test for *Acinetobacter baumannii* due to the high VME rates observed in these isolates.[7] ArıcıN et al.[19] also reported categorical and essential agreement rates of 93.7% and 100%, respectively, for *P. aeruginosa* isolates, with no VME or ME detected. In our study, colistin susceptibility in *P. aeruginosa* was associated with very low error rates when assessed using the CBDE method, in contrast to *Acinetobacter cbc*, where slightly elevated error rates, including very major errors, were observed.

The findings of our study for both Enterobacterales and non-fermentative isolates were highly satisfactory, demonstrating robust essential and categorical agreements. Despite an 8% error rate, with only 2% classified as very major errors, our results still support the CBDE method as a reliable tool for assessing susceptibility to polymyxins. Therefore, in clinical settings with a high sample load, CBDE presents a practical and reliable alternative for colistin MIC testing, offering a simpler method compared to rBMD.

Conclusion

The necessity for an enhanced method of determining colistin minimum inhibitory concentration (MIC) is pressing, given the rising incidence of multidrug-resistant gram-negative bacterial infections with limited therapeutic options. Presently endorsed techniques for assessing colistin susceptibility, such as agar dilution or broth microdilution (BMD) testing, are labour-intensive and demand expertise, time, and specific setup. The colistin broth disk elution (CBDE) method may address many of these challenges, offering a practical yet accurate and reproducible alternative for colistin MIC testing suitable for laboratories of varying capacities.

Limitations of the study

One limitation of our study is the limited number of test isolates, a constraint that arose due to financial considerations. This limitation may account for the slightly elevated error rates observed in our study compared to others. Nonetheless, the findings of our research are promising. With adequate financial support, the study could be expanded in the future to a multicentric design, thereby providing more precise data on colistin susceptibility.

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