Research Article

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Comparative evaluation of various disc elution methods for the detection of colistin-resistant gram-negative bacteria

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Abstract

Objectives: The current study was designed to determine the performance of colistin broth disc elution (CBDE), colistin broth micro elution (CBME), and microplate elution (MPE) methods with the broth microdilution (BMD) reference method.

Methods: For the study, multidrug-resistant Gram-negative bacilli (MDR GNB) isolates (n=715) obtained from various clinical specimens were tested for colistin sensitivity testing using CBDE, CBME, and MPE methods, and the results were compared with the BMD reference method.

Results: Of the total MDR-GNB isolates (n=715), 6.83 % (n=49) were colistin-resistant, while none yielded the *mcr* gene. The CBDE method demonstrated a sensitivity (95.91 %), specificity (100 %), positive predictive value (PPV) of 100 %, and negative predictive value (NPV) of 99.7 % when compared to the reference BMD method. The CBME method yielded 93.87 %, 93.33 %, 51.11 %, and 99.7 % of sensitivity, specificity, PPV, and NPV against the reference BMD method. However, the MPE method demonstrated sensitivity (91.83 %), specificity (92.64 %), PPV (47.36 %), and NPV (99.32 %), respectively, when compared to the reference BMD method.

Conclusions: The CBDE method has the potential to replace the BMD method for detecting colistin resistance among Gram-negative bacteria in laboratories. It offers a cost-effective and easy-to-learn alternative, while ensuring strong sensitivity and specificity compared to the BMD reference method.

Keywords: antimicrobial resistance; colistin resistance testing; colistin broth disc elution

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Introduction

Overuse and misuse of antibiotics have led to an alarming rise in drug resistance within bacterial infections, resulting in prolonged illnesses, higher mortality rates, and increased healthcare expenses worldwide. This increase in multidrug resistance (MDR) is an inevitable outcome of bacterial evolution and presents a significant public health threat. Consequently, colistin has been reevaluated, once considered a last-resort treatment. However, the extensive use of colistin has increased concerns about developing further resistance. Colistin is a potent panta-cationic antimicrobial agent that targets Gram-negative pathogens by binding to lipopolysaccharides (LPS) in their outer membrane. This interaction disrupts the membrane's integrity, resulting in bacterial cell death. Despite its efficacy, colistin was infrequently used due to its significant neurotoxic and nephrotoxic effects [1, 2].

Nonetheless, over the past decade, its use has surged threefold in combating infections caused by MDR bacteria. Concurrently, the unregulated use of colistin has resulted in the development of colistin-resistant strains [3, 4].

The spread or emergence of colistin resistance among bacteria already resistant to other antibiotics could cause untreatable infections. Gram-negative bacteria (GNB) develop colistin resistance as an adaptive mechanism following *in vitro* exposure to colistin. It emerges due to the dissemination of plasmid-mediated *mcr* genes or chromosomal mutation of genes associated with LPS synthesis. The most frequent resistance mechanism involves modifying the bacterial outer membrane by altering the structure of LPS [3, 5]. Other mechanisms may be caused by overexpression of efflux pumps or the excessive production of capsular polysaccharides. Clinical isolates have shown colistin resistance in GNB isolates such as *Enterobacteriales*, *Pseudomonas* species, and *Acinetobacter* species, whereas *Proteus*, *Morganella*, and *Providencia* species are naturally resistant to colistin [4]. It is important to assess colistin-resistant isolates to prevent their dissemination and treatment of MDR-GNBs.

Various culture media-based diagnostic tests, such as CHROMagar COL-APSE (Colistin-resistant A*cinetobacter*, *Pseudomonas*, *Stenotrophomonas*, and *Enterobacteriaceae spp*.), LBJMR Medium, Superpolymyxin, and other automated minimum-inhibitory concentration (MIC) based methods, such as Com ASP colistin, UMIC colistin kit, Vitek-2 Compact, Micronaut-s colistin broth, have been developed for the detection of colistin-resistant bacteria [6–8]. However, these methods are relatively expensive and may not be affordable in resource-limited settings. Further, colistin sensitivity testing using the disc diffusion method is not recommended because of the large molecular size of colistin, which prevents adequate diffusion through an agar medium. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Testing Standard Institute (CLSI) guidelines endorsed the micro broth dilution (BMD) test as the reference standard for the detection of colistin resistance [6, 9]. Further, various elution methods have been designed to identify colistin resistance [7, 8]. However, to our knowledge, there are no studies available to determine the performance of colistin broth disc elution (CBDE), colistin broth micro elution (CBME), and microplate elution (MPE) methods with the BMD for the detection of colistin resistance. Thus, this study aimed to analyze the accuracy of CBDE, CBME, and MPE methods with the reference BMD method in detecting colistin resistance in clinical bacterial isolates.

Materials and methods

Study settings

Clinical specimens and bacterial isolates

A cross-sectional study was conducted at the Department of Microbiology, M.M. Institute of Medical Sciences and Research, Ambala, India, between May 2021 and February 2022. In this study, MDR GNB (n=715) isolated from different clinical samples were subjected to CBDE, CBME, MPE, and BMD methods to assess colistin resistance in these isolates.

In the current study, MDR organisms were defined as bacteria resistant to a minimum of one antibiotic from three different classes of first-line antibiotics [10]. Additionally, the organisms with intrinsic colistin resistance, such as *Morganella morganii*, *Proteus*, *Providencia*, and *Serratia* species, were excluded. Further, we excluded duplicate samples, including repetitions or those collected from the same patients in the current study.

Bacterial identification, antimicrobial susceptibility testing, and MIC determination

The identification of GNBs was carried out using the Vitek-2 Compact system (bioMérieux, Marcy-l'Étoile, France). For all the GNBs, the MIC of various antibiotics was determined using the Vitek-2 Compact system with AST-N280 and AST-N281 cards, corresponding to lactose and non-lactose fermenters, respectively, according to the manufacturer's instructions. All MDR-GNB strains were further confirmed using the Kirby-Bauer disc diffusion method, with the AST results interpreted as per CLSI 2020 guidelines [9, 11]. Further, all elution tests were evaluated by two trained, independent technicians to ensure consistency, with each technician interpreting the results in a blinded manner. If any inter-observer variability was observed, the results were reviewed and resolved through joint review by the study supervisor.

Phenotypic detection of colistin resistance

Broth microdilution method for colistin

The colistin MIC was determined using colistin sulphate salt (Hi Media, Mumbai, India), which was dissolved in cation-

adjusted Mueller Hinton broth (CA-MHB, HiMedia, Mumbai, India) and distributed into untreated (50 µL) polystyrene microtiter plates with 96 wells. The standard BMD methodology (MIC range: 0.5–16 µg/mL) assessed the MIC values following CLSI standards [9, 11]. For *Enterobacterales*, *Pseudomonas aeruginosa (P. aeruginosa)*, and *Acinetobacter baumannii (A. baumannii)*, isolates with colistin MIC ≥4 µg/mL are considered resistant, while isolates with ≤2 µg/mL are considered intermediate [9, 11]. *Proteus mirabilis* (Colistin MIC >16 µg/mL) strain served as a positive control strain, and *Escherichia coli* (*E.coli*) ATCC 25922 was employed as the negative control.

Colistin broth disc elution method

For this test, four test tubes containing 10 mL of CA-MHB (HiMedia, Mumbai, India) were prepared and labeled 1 through 4, respectively. Colistin discs (10 µg colistin sulfate, Oxoid Ltd, UK) were added to the tubes to achieve final concentrations of 0 (growth control), 1, 2, and 4 µg/mL by adding 0, 1, 2, and 4 discs into the respective tubes. To allow colistin to elute from the discs into the broth, the tubes were incubated at room temperature for 30 min. A bacterial suspension was prepared in normal saline from the growth on blood agar and calibrated with 0.5 McFarland standard. From this suspension, 50 µL was added to each test tube and mixed thoroughly [7]. Afterward, the tubes were incubated at 37 °C for 24 h, after which they were visually examined for turbidity to determine the minimum inhibitory concentration [11].

Colistin broth micro elution method

The CBME method was performed by preparing four sterile test tubes containing 10 mL of CA-MHB (HiMedia, Mumbai, India) each. Colistin discs (10 µg colistin sulphate, Oxoid, UK) were added to the tubes in increasing numbers: 0 discs (growth control), 1, 2, and 4 discs, respectively. To allow the antibiotic to elute from the discs into the broth, the tubes were incubated at room temperature for 30 min, achieving final concentrations of 0, 1 µg/mL, 2 µg/mL, and 4 µg/mL, respectively. From each tube, 1 mL of the antibiotic-containing broth was transferred to new four sterile test tubes. A bacterial inoculum was prepared in normal saline using growth from a blood agar plate, and the inoculum was standardized with a 0.5 McFarland standard. Then, 5 µL of the standardized bacterial suspension was added to each tube, thoroughly mixed, and these tubes were incubated at 37 °C for 24 h [8]. After 24 h of incubation, the MICs were visually determined, and the results were interpreted using CLSI breakpoints [11].

Microplates elution method

The colistin-containing CA-MHB solution was distributed into microtiter plate wells, with 200 µL in each well, to establish concentrations of 0, 1, 2, and 4 µg/mL. To each well, including the growth control well (0 µg/mL), 3 µL of bacterial suspension, standardized to 0.5 McFarland, was added, followed by incubation at 37 °C for 24 h [8]. The MIC values were visually determined and interpreted according to CLSI breakpoints [11] (Table 1).

Genotypic detection

Detection of *mcr* genes using PCR

DNA templates were prepared using thermal cell lysis from overnight agar cultures. PCR amplification was performed as described by Rebelo et al. [12, 13]. The amplified products were analyzed by electrophoresis on a 1.5 % agarose gel to visualize the results, and staining was done by ethidium bromide at 130 V [12].

Data collection and statistical analysis

Diagnostic accuracy performance metrics such as sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), categorical agreement (CA), major errors (ME), and very major errors (VME) of BMD (reference methods) were calculated and compared with CBDE, CBME, and MPE as well. Additionally, to minimize the increased risk of Type I error, multiple testing corrections using the Bonferroni adjustment were also performed. The statistical significance of differences in sensitivity rates was evaluated

Table 1: Targeted genes and their corresponding primers.

Sr. No.	*mcr* genes	Size of amplicon, bp	Primer sequences (5′- 3′)
1.	*mcr* 1	320bp	fw AGTCCGTTTGTTCTTGTGGC rev AGATCCTTGGTCTCGGCTTG
2.	*mcr* 2	700bp	fw CAAGTGTGTTGGTCGCAGTT rev TCTAGCCCGACAAGCATACC
3.	*mcr* 3	900bp	fw AAA-TAAAAATTGTTCCGCTTATG rev AATGGAGATCCCCGTTTTT
4.	*mcr* 4	1100bp	fw TCACTTTCATCACTGCGTTG rev TTGGTCCATGACTACCAATG
5.	*mcr* 5	1644bp	fw ATGCGGTTGTCTGCATTTATC rev TCATTGTGGTTGTCCTTTTCTG

using McNemar's test. The statistical analysis of differences in colistin resistance detection rates was performed using the Chi-square test. GraphPad Prism 6 software (Graph-Pad Software, Inc., La Jolla, CA, USA) was used to determine the significance.

Ethical details

The study received approval from the Institutional Ethical Committee via letter no. MMIMSR/IEC/1916.

Results

A total of 715 MDR Gram-negative bacterial isolates were obtained from various clinical specimens. The highest number of isolates were obtained from urine samples (26.0 %, n=186), followed by pus (21.6 %, n=154), blood (16.7 %, n=119), sputum (18.0 %, n=129), wound swabs (9.5 %, n=68), vaginal swabs (2.0 %, n=14) and other specimens (6.6 %, n=47), respectively. Of the 715 MDR Gram-negative bacterial isolates tested for colistin resistance, 6.85 % (49/715) isolates showed colistin resistance by the BMD method and 6.57 % (47/715) by the CBDE method. These isolates primarily consisted of four species, with *Klebsiella pneumoniae* being the most prevalent, comprising 379 isolates; 3.43 % (13/379) of these were resistant to colistin by both BMD and CBDE methods. *E. coli* accounted for 196 isolates, with 5.1 % (10/196) isolates demonstrating resistance by both BMD and CBDE methods, respectively. However, *P. aeruginosa* included 101 isolates, with 17.82 % (18/101) resistant by BMD and 16.83 % (17/101) by CBDE methods, respectively. Finally, *A. baumannii* had 39 isolates, with 20 % (8/39) resistant by BMD and 17.95 % (7/39) by CBDE methods, respectively. Figure 1 illustrates a flowchart showing the study profile. Figure 2 shows the BMD method used for detecting colistin resistance. The MIC values of all the isolates tested against colistin (ranging from ≤0.5 µg/mL to 16 µg/mL) were noted and illustrated in Table 2. Most of the colistin-resistant GNBs showed MIC values of 4 µg/mL, followed by 8 µg/mL and 16 µg/mL, respectively. Non-lactose-fermenting bacteria had a higher rate of colistin resistance compared to lactose-fermenting bacteria. Among non-lactose fermenters, *P. aeruginosa* (18/49) isolates were found to be predominant colistin-resistant bacteria, followed by *A. baumannii* (8/49). However, among lactose fermenters, *K. pneumoniae* demonstrated the highest rate of resistance to colistin (13/49), followed by *E. coli* (10/49).

The antibiotic sensitivity results of all isolates tested using CBDE, CBME, and MPE methods were compared with the BMD method. The CBDE methods failed to detect two

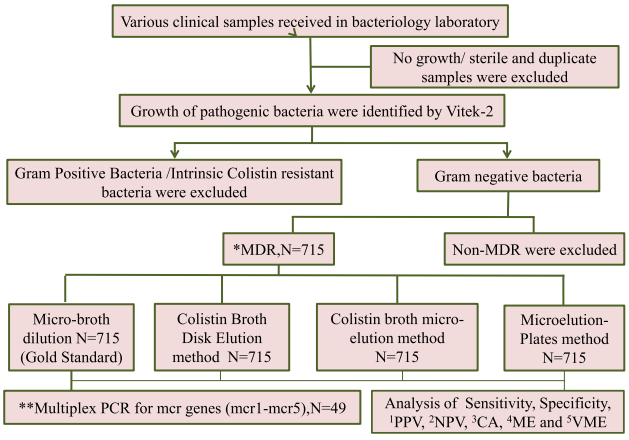

Figure 1: Flowchart showing the study profile.

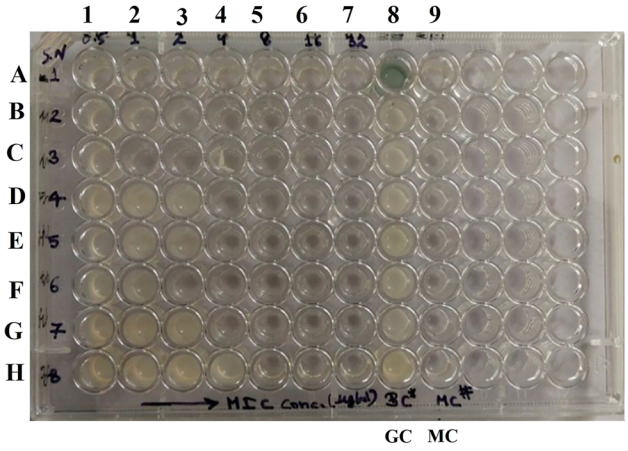

Figure 2: Broth microdilution (BMD) plate method for colistin susceptibility testing. Figure 2 Illustrates the BMD method in a 96-well microtiter plate. Row A (wells 1–9) serves as the negative control, while rows B–G (wells 1–8) are inoculated with the test organisms. Row H (wells 1–8) contains the positive control strain. Column 8 (wells A–B) is designated as the growth control, and column 9 (wells A–B) serves as the media control. Two-fold serial dilutions of colistin sulfate, ranging from 0.5 to 32 µg/mL (left to right), are prepared across the plate to determine the minimum inhibitory concentration (MIC). The MIC is defined as the lowest concentration of colistin at which no visible bacterial growth was observed.

colistin-resistant isolates, while the CBME and MPE methods identified 41 and 45 additional isolates as colistin-resistant, respectively. Among lactose-fermenting Gram-negative bacteria, the CBDE method showed antibiotic susceptibility patterns comparable to the BMD (the reference method), while both the CBME and MPE methods showed minor variations in identifying colistin-sensitive bacteria, as shown in Table 3. However, the colistin susceptibility of the non-lactose fermenting bacteria tested showed variations in all the elution methods evaluated. Figure 3 shows the

Table 2: Distribution of colistin minimum inhibitory concentrations in gram-negative bacteria by the broth microdilution method.

Total number of gram-negative bacterial isolates	Minimum inhibitory concentration values of colistin					
	≤0.5 µg/mL	1 µg/mL	2 µg/mL	4 µg/mL	8 µg/mL	16 µg/mL
Klebsiella pneumoniae (n=379)	354 (93.4 %)	7 (1.8 %)	5 (1.31 %)	9 (2.3 %)	3 (0.7 %)	1 (0.2 %)
Escherichia coli (n=196)	172 (87.7 %)	8 (4.08 %)	6 (3.06 %)	6 (3.06 %)	4 (2.04 %)	00
Pseudomonas aeruginosa (n=101)	56 (55.44 %)	21 (20.7 %)	6 (5.9 %)	17 (16.83 %)	1 (0.99 %)	00
Acinetobacter baumannii (n=39)	15 (38 %)	14 (35.8 %)	2 (5.9 %)	7 (17.94 %)	1 (2.5 %)	00
Total (n=715)	597 (83.5 %)	50 (7 %)	19 (2.6 %)	39 (5.45 %)	9 (1.25 %)	1 (0.13 %)

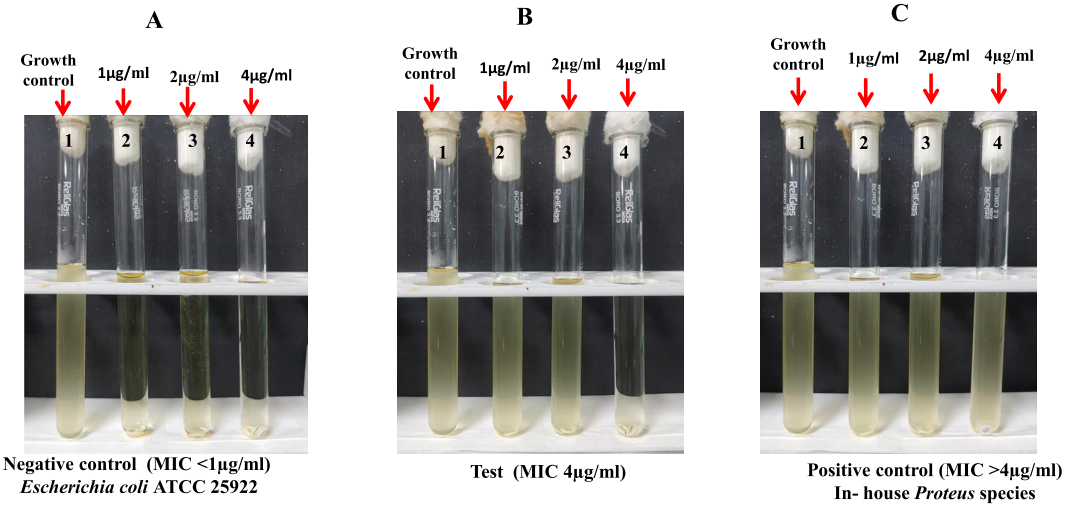

Figure 3: Colistin broth disc elution method for detection of colistin resistance in gram-negative bacilli. From left to right: (A) Negative control consisting of four tubes – first tube with cation-adjusted Mueller–Hinton broth (CAMHB) inoculated with the colistin-susceptible *Escherichia coli* (ATCC 25922) strain serving as the growth control, and three tubes containing CAMHB and same *E. coli* (ATCC 25922) strain with colistin at 1, 2, and 4 µg/mL, all showing no visible growth, indicating colistin susceptibility; (B) test isolate, with four tubes containing CAMHB and the test strain, yielding a minimum inhibitory concentration (MIC) of 4 µg/mL (visible growth at ≤ 2 µg/mL, no growth at 4 µg/mL defines the MIC); (C) positive control (colistin-resistant in-house *Proteus* species), showing visible growth in tubes containing 1, 2, and 4 µg/mL of colistin indicating colistin resistance.

identification of colistin resistance by the CBDE method. The comparison of the susceptibility pattern of all the MDR-GNB tested against colistin is depicted in Table 3.

Figure 4 illustrates the identification of colistin-resistant GNB by the CBME method. The distributions of colistin MICs for all MDR-GNB were analyzed and compared across all tested elution methods, as shown in Table 3. Figure 5 shows the identification of colistin-resistant GNB by the MPE method. The performance of the CBDE method demonstrated minor variations in MIC detection when compared with the BMD reference method, while both the CBME and MPE methods showed significant variations ($p<0.0001$), as presented in Table 4.

The diagnostic accuracy of all the elution methods was evaluated and compared with the BMD method on parameters such as sensitivity, specificity, PPV, NPV, M.E, VME, and CE, and the details are depicted in Table 5. A comparative bar chart showing sensitivity, specificity, PPV, and NPV for each method (with standard error) is presented in Figure 6. The major errors were noted in the CBM method (6.60 %) and MPE method (7.35 %), respectively. However, no major errors were noted in the CBDE method compared to BMD. The categorical agreement exceeded 90 % for all methods tested in the current study. The results demonstrated that among the tested methods, CBDE showed superior performance with a

Table 3: Comparison of colistin resistance rates among gram-negative bacterial isolates as determined by broth microdilution, colistin broth disc elution, colistin broth microelution, and microplate elution methods with multiplex PCR screening for *mcr*-1 to *mcr*-5 genes.

Total number of bacterial isolates (n=715)	[a]BMD (n=715) [e]R/I, R %	[b]CBDE (n=715) [e]I/R, R %	[c]CBME (n=715) [e]I/R, R %	[d]MPE (n=715) [e]I/R, R %	Multiplex polymerase chain reaction for the detection of *mcr (1–5) genes*
Klebsiella pneumoniae sp.	13/366	13/366	32/347	35/344	Not detected
(n=379)	(3.4 %)	(3.4 %)	(8.44 %)	(9.23 %)	
Escherichia coli	10/186	10/186	22/174	22/174	-do-
(n=196)	(5.1 %)	(5.1 %)	(11.22 %)	(11.22 %)	
Pseudomonas aeruginosa	18/83	17/84	25/76	26/75	-do-
(n=101)	(17.82 %)	(16.83 %)	(21.75 %)	(16.83 %)	
Acinetobacter baumannii	8/31 (20.5 %)	7/32	11/28	11/28	-do-
(n=39)		(17.94 %)	(28.20 %)	(28.20 %)	
Total (715)	49/666	47/668	90/625	94/621	Not detected
	(6.83 %)	(6.57 %)	(12.58 %)	(13.14 %)	

[a]Broth microdilution method, [b]Colistin broth disc elution method, [c]Colistin broth micro elution method, [d]Microplate elution method, [e]I-Intermediate, R-Resistant, R %-resistance percentage.

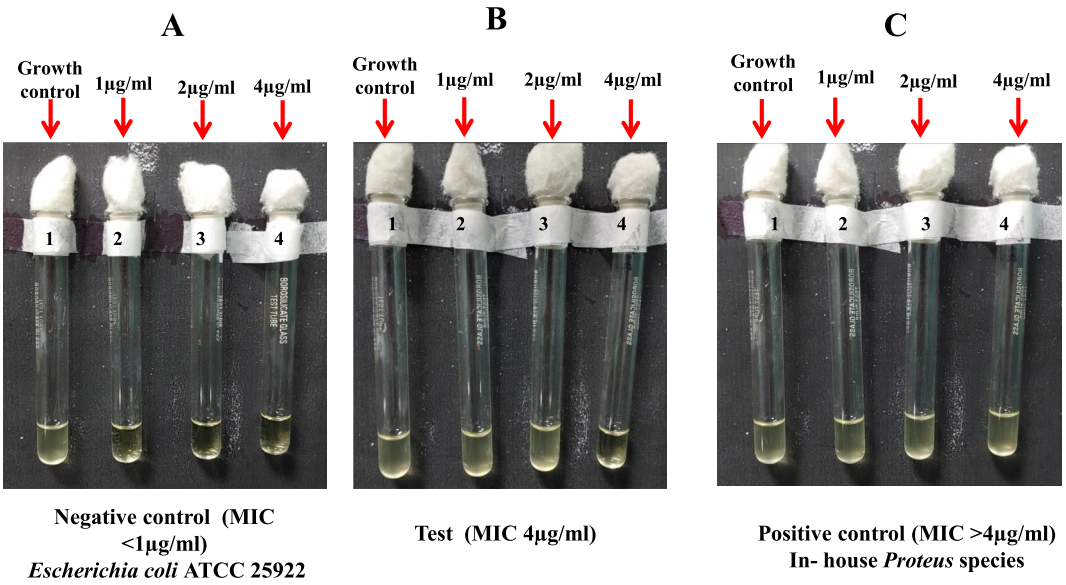

Figure 4: Colistin broth micro-elution method for detection of colistin resistance in gram-negative bacilli. From left to right: (A) Negative control with four tubes – first tube containing cation-adjusted Mueller–Hinton broth (CAMHB) inoculated with colistin-susceptible *Escherichia coli* (ATCC 25922) serving as the growth control, and three tubes containing the same strain with colistin at 1, 2, and 4 µg/mL, all showing no visible growth, thereby confirming susceptibility; (B) test isolate as represented by four tubes with CAMHB and the test strain, showing a minimum inhibitory concentration (MIC) of 4 µg/mL (growth present at ≤ 2 µg/mL but absent at 4 µg/mL); (C) positive control, an in-house colistin-resistant Proteus species with visible growth at 1, 2, and 4 µg/mL, indicating resistance to colistin.

sensitivity (95.91 %) and specificity (100 %) compared to the reference BMD method (McNemar test, p<0.005) in detecting colistin resistance. Similarly, the CBME achieved a sensitivity (93.87 %) and specificity (93.33 %) when compared to the BMD method (McNemar test, p=0.0574). However, the MPE method showed slightly lower but still substantial performance, with a sensitivity (91.83 %) and specificity (92.64 %) compared to the BMD method (McNemar test, p=0.063). Additionally, molecular analysis (multiplex PCR) of all colistin-resistant GNBs yielded negative results for *mcr* genes (Table 3). Table 6 shows the comparison of the cost analysis of CBDE, CBME, and the MPE method with the BMD reference method.

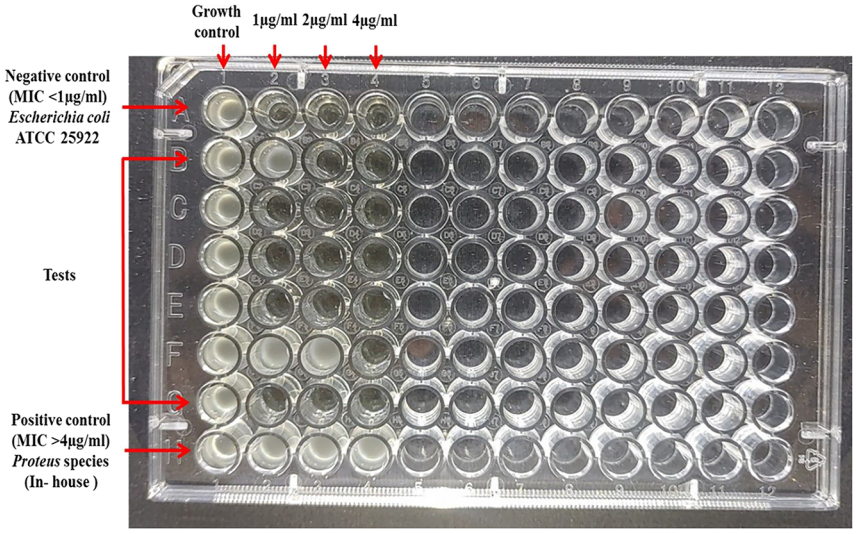

Figure 5: Microplate elution plate method for detection of colistin resistance in gram-negative bacilli. From left to right, (row A): Negative control consisting of four wells – the first well containing cation-adjusted Mueller–Hinton broth (CAMHB) inoculated with colistin-susceptible *Escherichia coli* ATCC 25922 as the growth control, and three wells containing the same strain with colistin at 1, 2, and 4 µg/mL, all showing no visible growth, confirming susceptibility. (rows B-G): test isolates dispensed across the different concentration range and showing colistin susceptibility and resistance depending on the strain. (row H): positive control, an in-house colistin-resistant Proteus species, showing visible growth at 1, 2, and 4 µg/mL, consistent with colistin resistance. Column 1: growth control (0 µg/mL colistin). Columns 2–4: 1, 2, and 4 µg/mL colistin, respectively.

Table 4: Comparison of minimum inhibitory concentrations as detected by various antimicrobial susceptibility test methods.

[a]MICs of colistin	[b]BMD method (n=715)	[c]CBDE method (n=715)	[d]CBME method (n=715)	[e]MPE method (n=715)	p-Value
≤ 1 µg/mL	647	651	556	576	P<0.0001[f]
2 µg/mL	19	17	69	45	
4 µg/mL	14	38	43	59	
≥ 4 µg/mL	0	09	47	35	

[a]Minimum inhibitory concentrations, [b]Broth microdilution method, [c]Colistin broth disc elution method, [d]Colistin broth micro elution method, [e]Microplate elution method, [f]Chi-square test.

Discussion

In recent years, carbapenem-resistant and MDR-GNBs have been increasingly isolated from clinical samples. Colistin is increasingly used as a treatment option against MDR and carbapenem-resistant GNBs [6]. However, resistance to colistin has been detected in various GNBs across multiple countries, making the timely detection of colistin resistance critical for patient care. Different methods have been introduced for detecting colistin resistance, including quick assays, disc diffusion tests, E-test methods, and the BMD reference method. However, all of these techniques require significant resources, specialized equipment, and expertise; such limitations can affect their availability and broad implementation, particularly in low-resource settings. These challenges highlight the urgent need for a simple, reliable, cost-effective alternative method that can be implemented in resource-limited settings. This study evaluated the performance of various elution methods (CBDE, CBME, and MPE) with the BMD method. This study compared diagnostic accuracy parameters such as sensitivity, specificity, NPV, and PPV of various elution methods with the BMD method. Additionally, we assessed the ease of implementation and cost-effectiveness of these elution methods with the BMD method [14, 15].

Of the 715 MDR-GNB analyzed in this study, *K. pneumoniae* was the most prevalent GNB (53 %), with *E. coli* (27.4 %), *P. aeruginosa* (14.1 %), and *A. baumannii* (5.4 %) following. This high burden of MDR strains in the hospital setting underscores the need for proper infection control measures, targeted antibiotic policies, and structuring hospital-specific antimicrobial stewardship programs. According to the current EUCAST (2025) guidelines, colistin sensitivity is defined as MIC ≤ 2 µg/mL, with resistance classified as MIC >2 µg/mL for both *Enterobacterales* and *P. aeruginosa* [16]. However, the CLSI guidelines define colistin resistance breakpoints for *P. aeruginosa* and *Acinetobacter* spp. are set at ≥ 4 µg/mL, with susceptibility defined as ≤ 2 µg/mL, but do not provide breakpoint interpretations for Enterobacterales [17]. It is evident from Table 1 that 83.5 % of highly susceptible isolates suggest colistin remains an effective option for most infections in this population. However, 7 and 2.6 % of isolates with MIC=1 µg/mL and 2 µg/mL indicate emerging resistance trends. The 6.83 % of resistant isolates (MIC ≥ 4 µg/mL) are clinically significant because colistin is often considered a last-line treatment option for MDR infections, and these patients require combination therapies with rifampin, carbapenems, or novel agents like cefiderocol [18]. Analysis of colistin resistance

Table 5: Diagnostic performance of the various elution methods with the broth microdilution reference method.

Methods (n=715)		[a]BMD method		Sensitivity (%) (95 % CI)	Specificity (%) (95 % CI)	PPV (%) (95 % CI)	NPV (%) (95 % CI)	Major errors, %	Very significant errors, %	Categorical agreement, %	McNemar test p-Value (bonferroni adjusted)
		Positive	Negative								
[b]CBDE method	Positive	47	0	95.91 (84.86–99.2)	100 (99.28–100)	100 (90.58–100)	99.7 (98.8–99.94)	00	4.08	99.72	**0.015**[a]
	Negative	2	666								
[c]CBME method	Positive	46	44	93.87 (82.13–98.4)	93.33 (91.16–95.1)	51.11 (40.1–61.7)	99.52 (98.48–99.87)	6.60	6.12	93.42	0.172
	Negative	03	622								
[d]MPE method	Positive	45	49	91.83 (79.51–97.35)	92.64 (90.32–94.45)	47.36 (37.55–58.36)	99.32 (98.24–99.79)	7.35	8.16	92.58	0.189
	negative	04	617								

[a]Broth microdilution method, [b]Colistin broth disc elution method, [c]Colistin broth micro elution method, [d]Microplate elution method. Bold values indicate statistically highly significant.

patterns across bacterial species (Table 1) revealed that only a single *K. pneumoniae* isolate (0.01 %) showed a high degree of resistance to colistin with a MIC value of 16 µg/mL. This degree of resistance makes colistin ineffective, significantly limiting available therapeutic options for this isolate. Further, among colistin-resistant isolates (MIC of > 4 µg/mL), *P. aeruginosa* was the most frequent (n=18), followed by other GNBs, respectively (Table 4). This potential cause of colistin resistance could be linked to the environmental persistence of colistin-resistant strains in ICUs and hospital settings [14, 18, 19].

Colistin resistance detection is a major concern worldwide, particularly in low-resource settings. The CBDE method to detect colistin resistance by Simner et al. was a breakthrough [7]. The other modification of the CBDE method was accomplished by Dalmolin et al. in 2020 [8]. As per the World Health Organization Global Antimicrobial Resistance and Use Surveillance System, it is advised that all carbapenem-resistant bacteria and MDR-GNB be tested for colistin resistance. In India, we are still in urgent need of a method to assess colistin resistance among MDR-GNB on a routine basis to replace the BMD method [20]. For this study, we evaluated available elution methods, and the results were evaluated with the reference BMD method. The current study demonstrated a zero percent discrepancy in the detection of colistin resistance in fermenters by the CBDE method compared to the BMD reference method. However, in the case of non-fermenting bacteria, the CBDE method failed to detect colistin-resistant strains in one *P. aeruginosa* and one *A. baumannii* isolate, respectively. This may be due to various reasons, such as the lower sensitivity of the CBDE method to detect colistin resistance in comparison with the BMD reference method. The other possible reason would be the alteration of LPS through chromosomal mutations, resulting in reduced colistin binding affinity [21]. However, it is evident from Table 2 that both CBME and MPE methods detected a few false-positive colistin-resistant GNBs and failed to identify some false-negative colistin-resistant bacteria as well. These false positive results may be due to certain bacterial strains' inherent ability to modify their colistin binding sites, thereby affecting the accuracy of resistance detection [7, 8, 21]. Further, the failure of detection of false-negative colistin resistance may be attributed to the fact that if the proportion of resistant cells is too low compared to the total population, this type of colistin heteroresistance is largely undetected [22].

Colistin disc elution methods were developed to assess colistin resistance in bacteria and provide MICs comparable to the BMD method [7]. In the current study, the data of MICs as obtained by the BMD method were compared with the CBDE, CBME, and MPE methods, and variations were noted.

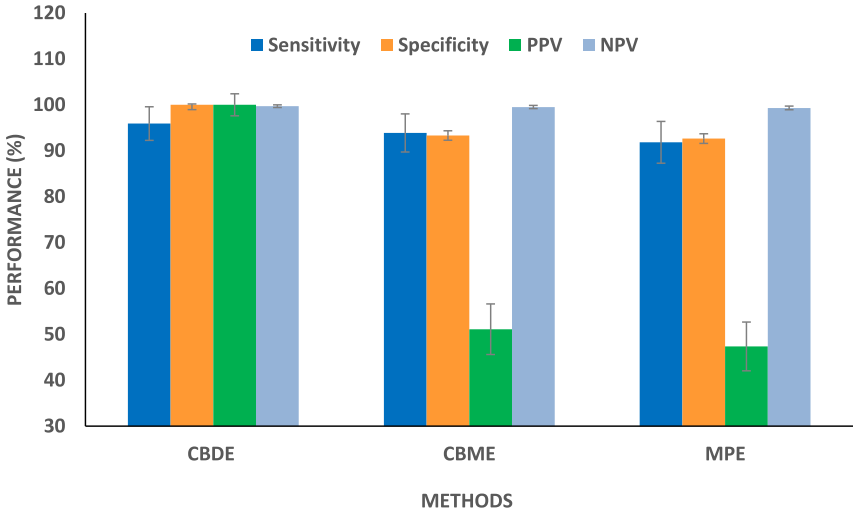

Figure 6: Comparison of performance by various disc elution methods, such as colistin broth disc elution method (CBDE), colistin broth micro elution method (CBME), and microplate elution method (MPE) with broth microdilution method (BMD) for the detection of colistin resistance.

Table 6: Comparison of cost analysis of colistin broth disc elution, colistin broth micro elution, and microplate elution method with the broth microdilution reference method (the amount is provided in both Indian rupees and US dollars).

Reagents and labor requirements	Broth micro-dilution method		Colistin broth disc elution method		Colistin broth micro elution method		Micro-plate elution method	
	₹	$	₹	$	₹	$	₹	$
Colistin disc or solution	15	0.20	30	0.40	10	0.13	10	0.13
Culture media (like cation-adjusted Mueller-Hinton broth)	30	0.40	10	0.13	5	0.07	10	0.13
Consumables (plates, tubes, cotton)	25	0.33	10	0.13	15	0.20	25	0.33
Labor requirements (technicians and others)	50	0.67	30	0.40	40	0.53	50	0.67
Total estimated cost per test	120	1.6	80	1.06	70	0.93	95	1.27

MICs were categorized from ≤1 µg/mL to ≥4 µg/mL. The elution method could not be used to detect ≤0.5 µg/mL and ≥4 µg/ml MICs of colistin; therefore, we compared only ≤1 µg/mL to ≥4 µg/mL MICs. This narrow MIC range (1–4 µg/mL) in disc elution methods can further limit the detection of isolates with high-level resistance, particularly when the actual MIC exceeds the upper limit of the test range. This may further result in underestimation of resistance rates. Additionally, the visual interpretation of MIC endpoints involves a degree of subjectivity, mainly due to observer bias. To minimize this variability, two independent technicians performed all readings, and discrepancies were resolved through joint review by the study supervisor. However, the use of instrument-based readings or digital image analysis could minimize this observer bias. The CBDE method yielded consistent outcomes with the BMD reference method, whereas both the CBME and MPE methods showed varied MIC values, with many Gram-negative bacteria exhibiting higher MIC values. (Table 3). These variations in MIC values can be attributed to two main factors: either inconsistent colistin elution from the discs into the broth medium or inadequate mixing of colistin within the medium. Although we implemented quality control measures, including vortexing all tubes before sample processing, there are no currently available methods to validate the colistin concentration in these elution methods.

The diagnostic efficiency of the test is of utmost importance for its acceptance and implementation in routine laboratory analysis. We also determined their relative diagnostic performances, such as sensitivities, specificities, NPV, PPV, ME, VME, EA, and CA, and the details are summarized in Table 4. It is evident from Table 4 that the CBDE method showed higher sensitivity and specificity in comparison with the reference BMD method. Further, the McNemar test p-values for each method were reported as follows: CBDE ($p<0.005$), CBME ($p=0.0574$), and MPE ($p=0.063$), providing a clearer perspective on their performance relative to the reference BMD method. CBDE demonstrated statistically significant concordance with BMD, underscoring its reliability for detecting colistin resistance. While CBME and MPE showed slightly lower sensitivity and specificity and did not achieve conventional statistical significance, their performance remains robust, indicating that both methods are likely to be clinically meaningful and could serve as feasible alternatives in routine laboratory practice. Furthermore, the CBDE method showed no ME and a very low VME rate (Table 4), suggesting that CBDE is the most reliable method to substitute for the

BMD method in laboratory settings. In contrast, CBME and MPE methods displayed higher rates of VME (6.12 and 8.16 %, respectively), indicating resistant strains were incorrectly reported as susceptible (false-negative), which could result in inappropriate therapies and treatment failure. This type of treatment failure occurs mainly because of the administration of an ineffective drug instead of a potentially active alternative (due to false susceptibility), eventually resulting in prolonged infections, increased transmission rates of MDR strains within healthcare settings, and higher mortality rates. In contrast, tests with high major errors (ME), where susceptible isolates are incorrectly reported as resistant, can lead to the unnecessary avoidance of colistin and the use of less effective alternative treatments. Further, the relatively low PPV of CBME (51.11 %) and MPE (47.36 %) suggests high false-positive results. These results would mislead the treatment options in resource-limited settings as well. The CBDE, CBME, and MPE only provide data for MIC concentrations ranging from 1 µg/mL to 4 µg/mL, suggesting these tests may not be suitable for detecting colistin resistance at MIC values beyond this range. The calculation of EA (Essential Agreement) for disc elution tests was unsatisfactory and was excluded from further analysis in the current study. This suggests that the MIC determination using elution methods may not align well with BMD, limiting their consistency in certain cases. This effect was most evident among isolates with MIC values around the clinical breakpoint of 2 µg/mL, where even minor variability in elution often resulted in borderline strains being categorized as resistant. The contributing factors likely include inconsistent or incomplete drug release, binding of colistin to plasticware, and pipetting inaccuracies in low-volume assays. Additionally, minor variations in the composition of cation-adjusted Mueller–Hinton broth or residual substances from antibiotic discs may influence apparent colistin activity, with the greatest impact observed for isolates near the susceptibility breakpoint. However, future use of these assays should prioritize the strict standardization of the elution process to improve reproducibility. The improvement strategies may include establishing uniform elution times, using low-binding plasticware to reduce adsorption rate, ensuring thorough mixing of solutions just before inoculation, and quantitative validation of eluted concentrations of colistin using validated analytical methods. It is also advisable that laboratories consistently incorporate both susceptible and borderline control strains to track performance across batches, while confirming results in the 2–4 µg/mL range with the BMD method. These adjustments would reduce the likelihood of false-positive results, improve consistency, and strengthen the overall reliability of CBME and MPE as a practical alternative to the BMD reference method.

In the current study, all the colistin-resistant Gram-negative bacteria were screened by multiplex PCR for all *mcr* genes yielded negative results. However, these findings have limited statistical significance because the resistance to colistin can develop through two mechanisms: the spread of *mcr genes* (plasmid-mediated) or chromosomal mutations in genes responsible for LPS synthesis [3, 5]. Therefore, the lack of *mcr* genes does not limit the significance of this study. We also noted the ease of performing tests, their associated cost, and training requirements for each test in this study (Table 6). In resource-limited settings, performing BMD can be challenging because it requires specialized glass-coated plates, calibrated micropipettes, and well-trained personnel [23]. In these settings, disc-based methods such as CBDE, CBME, and MPE provide feasible and practical alternatives. For example, our cost analysis demonstrated that CBDE and CBME can be completed for approximately 0.8–1.0 USD per test, compared with 1.5–2.0 USD for BMD. Moreover, the simplicity of the CBDE protocol reduces the burden of training and minimizes handling errors, making it particularly suitable for regional and peripheral laboratories. By contrast, in high-income settings, BMD remains the reference standard, with many laboratories relying on automated MIC detection and molecular techniques to detect colistin resistance. In these contexts, elution-based assays are best positioned as supplementary tools, for example, a useful method to detect uncertain MIC results or as a low-cost preliminary screening method in epidemiological surveillance studies for screening of a large collection of bacterial isolates. Further, this study possesses a few limitations, for instance, a) In this study, molecular screening was restricted to the detection of *mcr-1* to *mcr-5* genes. Although mcr-6 to mcr-10 have been reported only rarely in various settings, these additional *mcr* variants were not assessed in our analysis and may therefore have remained undetected. b) Chromosomal resistance mechanisms such as pmrAB and mgrB mutations were not assessed due to the limited resources. c) Colistin concentrations were not quantitatively tested due to resource restrictions and were instead assumed using standard disc elution methods. d) Other rapid procedures, such as rapid NP and col Agar, should be evaluated for all the strains tested in this study.

In summary, the CBDE method demonstrated higher sensitivity and specificity while requiring the least amount of training compared to the BMD for detecting colistin-resistant GNBs, suggesting the potential of the CBDE

method to replace the BMD method for the routine identification of colistin-resistant GNBs in resource-limited laboratories.

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