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Original Article

Microbiology Section

Comparative Evaluation of Broth Microdilution, Disc Elution and Rapid Colistin NP Test for Detecting Colistin-resistant Enterobacterales: A Cross-sectional Study

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ABSTRACT

Introduction: As Antimicrobial Resistance (AMR) continues to pose a global health crisis, rapid and accurate antimicrobial susceptibility testing is crucial. In Intensive Care Unit (ICU) patients with sepsis, the 48-hour delay associated with conventional culture-based reports can be critical. The present study aimed to compare the rapid Colistin NP test with conventional culture techniques as a key strategy to combat antibiotic resistance caused by Multidrug Resistant (MDR) organisms.

Aim: To perform a comparative evaluation of three different methods for detecting colistin resistance: Broth Microdilution (BMD), Disc Elution (DE), and rapid colistin NP tests. in Enterobacterales And to determine the Minimum Inhibitory Concentration (MIC) of colistin using the BMD test, to determine the MIC of colistin.

Materials and Methods: The present cross-sectional study was conducted over twelve months (January 2023 to December 2023) at SRM Medical College Hospital and Research Centre, Chengalpattu, Tamil Nadu, India. The primary inclusion criteria that was set for the study was that blood specimens from ICU patients testing positive for Gram Negative Bacilli (GNB) were included in the study. The sample size was determined to be 178. Blood samples positive only for GNB belonging to Enterobacterales were taken into consideration. BMD and DE tests were performed to determine the MIC of colistin. Additionally, the rapid colistin NP Test was conducted to assess antibiotic susceptibility.The assessment was conducted directly from BacT/ALERT bottles as well as from bacterial isolates. The blood samples were collected from patients above 18 years of age. Statistical analysis was performed using IBM Statistical Package for Social Sciences (SPSS) software. The Chi-square test was used to assess the correlation between BMD (the gold standard method) and other methods such as DE and the rapid colistin NP Test (from both isolates and BacT/ALERT bottles). A p-value of <0.05 was considered statistically significant.

Results: Over the study period, 178 GNB isolates were identified. Of these, 151/178 (84.8%) were found to be colistin-sensitive by BMD and DE tests. Using the rapid colistin NP Test, 153/178 (85.9%) isolates from bacterial cultures and 154/178 (86.5%) from BacT/ALERT bottles were identified as colistin-sensitive and resistant, respectively. The sensitivity and specificity of the rapid colistin NP Test were 92.5% and 100% for bacterial isolates, and 88.9% and 100% for BacT/ALERT bottles, respectively.

Conclusion: The present study demonstrates that the rapid colistin NP test is an effective and reliable method for the early detection of colistin resistance in GNB within the enterobacterales group. The test showed high sensitivity and specificity, offering rapid results that can significantly aid clinical decision-making. Its implementation can facilitate timely initiation of appropriate antimicrobial therapy, helping to curb the spread of resistant strains and improve patient outcomes. These findings support the routine use of the rapid colistin NP Test in clinical microbiology laboratories for the prompt identification of colistin-resistant pathogens.

Keywords: Antibacterial agents, Antimicrobial susceptibility test, Gram-negative bacilli, Minimum inhibitory concentration

INTRODUCTION

Enterobacterales encompasses a diverse group of bacteria commonly found in the human intestinal tract, capable of causing various infections. This family includes numerous genera and species of bacteria. Some clinically significant genera include *Klebsiella*, *Escherichia coli*, *Enterobacter*, *Citrobacter*, *Salmonella*, *Shigella*, *Proteus*, and *Serratia*.

Many Enterobacterales bacteria are part of the normal microbial population in the human gastrointestinal tract. Despite being normal flora, these bacteria can act as opportunistic pathogens, causing infections such as Urinary Tract Infections (UTIs) that are caused by common organisms include *E. coli*, *Klebsiella*, and *Proteus*, Diarrhoea, which is often caused due to pathogens like certain strains of *E. coli* (e.g., enterotoxigenic *E. coli*), eye and skin infections caused mainly by some Enterobacterales members can cause conjunctivitis and skin infections, particularly in immunocompromised individuals, meningitis caused by certain Enterobacterales, such as *Klebsiella* and *Escherichia coli*, can cause meningitis, especially in neonates and immunocompromised patients, Pneumonia where *Klebsiella pneumoniae* is notorious for causing pneumonia, particularly in hospitalised patients [1].

Antibiotic resistance among these organisms is a growing concern, making treatment increasingly challenging. The most life-threatening infections caused by Enterobacterales are bloodstream infections, which have high mortality rates, especially in ICU patients [2].

Carbapenemase-producing Enterobacterales (CPE) is the most clinically significant MDR bacteria. Some older classes of antimicrobial drugs, like polymyxins (Colistin and Polymyxin B), were abandoned in the early 1970s due to their toxicity and severe side-effects. However, they have recently been reintroduced into clinical practice as a last-resort therapy for carbapenem-resistant bacterial infections [3].

MDR among bacteria has led to the ineffectiveness of antibiotics, resulting in prolonged hospital stays for patients. Certain subpopulations of Enterobacterales produce the enzyme beta-lactamase, which cleaves the central ring structure responsible for the efficacy of beta-lactam antibiotics, including carbapenems and cephalosporins [4]. Repeated exposure to these antibiotics creates selection pressure, further increasing drug resistance. Antibiotic resistance, particularly among Enterobacterales, poses a serious threat to public health due to the limited treatment options available. The increasing prevalence of CPE in clinical settings, especially among critically ill patients, highlights the urgency of understanding resistance mechanisms and evaluating alternative treatment options. The present study is necessary to address the gaps in effective antimicrobial therapy, understand the molecular basis of resistance, and inform evidence-based clinical management strategies for MDR infections. The study introduces a contemporary evaluation of the resurgence and effectiveness of older antibiotics like polymyxins (Colistin and Polymyxin B) against modern, MDR strains. It also explores recent resistance patterns, including beta-lactamase production, in the context of CPE. This dual focus on reintroducing old drugs and understanding current resistance mechanisms has not been widely addressed in previous research, making the study novel. The present study is unique because it combines clinical data on treatment outcomes in Intensive Care Unit (ICU) settings with microbiological analysis of resistance mechanisms, such as beta-lactamase activity, and the evaluation of last-line therapies (polymyxins) that were previously abandoned.

It bridges the gap between clinical challenges and laboratory-based insights to form a comprehensive understanding of MDR in enterobacterales. CPEs exhibit high levels of resistance to current frontline antibiotics, but selected reintroduced antimicrobials (e.g., polymyxins) may offer effective treatment alternatives when used appropriately, despite their toxicity. Understanding resistance mechanisms like beta-lactamase production will improve therapeutic outcomes and inform infection control strategies.

Antibiotics that were once effective now fail to eradicate these organisms [5]. Therefore, early detection of MDR is crucial for accurate and effective infection management. The key to appropriate therapy lies in identifying resistance patterns. However, conventional culture methods typically require 48 to 72 hours from specimen collection to produce results. This is the reason a comparative evaluation of BMD, DE and rapid colistin NP Test was executed for detecting colistin- resistant Enterobacterales. Moreover, for running the tests, all the blood samples were collected from ICU patients that flagged positive only for GNB belonging to enterobacterales. After flagging of the sample with Gram- negative bacilli, BMD and DE tests were executed for determining the colistin resistance among the isolates by observing the MIC. Then, rapid colistin NP test was performed both from isolates and from blood culture bottles directly to find out the antimicrobial susceptibility.

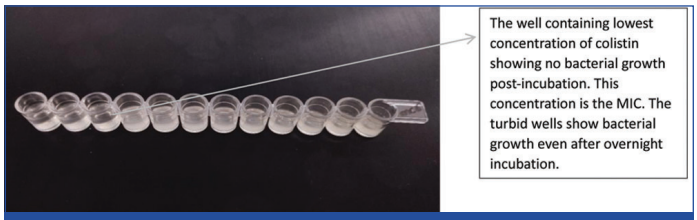
MATERIALS AND METHODS

The present cross-sectional study was conducted on 178 bacterial isolates belonging to enterobacterales in a span of one year (January 2023 to December 2023). All the samples were collected from patients admitted in ICU. Only samples flagging positive for Gram-negative bacilli were accepted for the study. No follow-up was done on the patient after collection of the sample. The study was executed at SRM Medical College Hospital and Research Centre, Kattankulathur, Tamil Nadu, India. Institutional Ethics Approval was obtained (SRMIEC-ST0123-288).

Inclusion and Exclusion criteria: Blood culture specimens from ICU patient flagged positive for GNB belonging to Enterobacterales were included in the study, while blood samples from non-ICU patients were excluded. To determine the MIC of colistin for these bacterial isolates, two phenotypic methods- BMD and DE tests-were performed [6,7]. NCTC 13846 *E. coli* (MIC: 4-8 µg/mL) and ATCC 25922 *E.coli* (MIC: 0.25-2 µg/mL) were used as positive and negative controls, respectively [8]. Additionally, antimicrobial susceptibility to colistin was determined using the RAPID COLISTIN NP test from bacterial isolates and from BacT/ALERT bottles.

Study Procedure

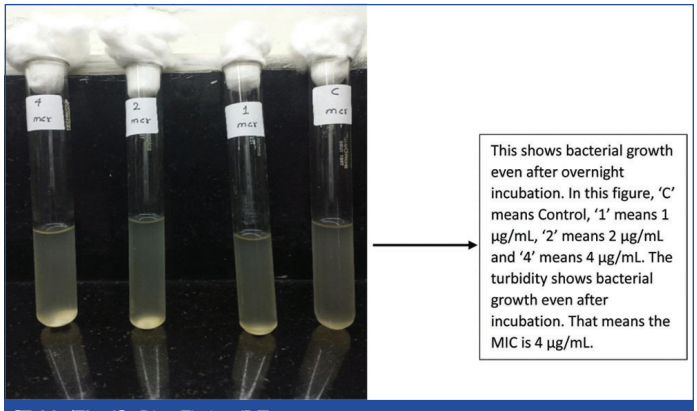
Broth Microdilution (BMD): Two to three well-isolated colonies were selected from a nutrient agar plate (not older than 24 hours). The required number of inoculum broth vials and sterile tips from the BMD kit were retrieved. The colonies were transferred to 5 mL of normal saline and mixed thoroughly to avoid clumps. Then, 50 µL of inoculated saline was added to the inoculum broth vial using a micropipette. This process was repeated for all samples. BMD strips (Microxpress, a division of Tulip Diagnostics (P) Ltd., a PerkinElmer company) were used. A 200 µL of inoculum broth was loaded into each well, and the strips were incubated overnight at 37°C. The MIC was determined as the lowest concentration of colistin that prevented visible bacterial growth [Table/Fig-1] [9,10].



[Table/Fig-1]: Broth Microdilution (BMD) for Colistin Resistance.

Disc Elution (DE): Three to five colonies were picked from a fresh non-selective agar plate and transferred to 5 mL of sterile saline. The solution was mixed well to prevent clumps, and the turbidity was adjusted to match the 0.5 McFarland standard. Colistin discs (10 µg) and Cation-Adjusted Mueller-Hinton Broth (CAMHB) tubes (10 mL each) were brought to room temperature before testing.

Four tubes were labeled: 1 µg/mL, 2 µg/mL, 4 µg/mL, and a control (C). One colistin disc was added to the 1 µg/mL tube, two discs to the 2 µg/mL tube, and four discs to the 4 µg/mL tube. No colistin discs were added to the control tube. The tubes were vortexed and incubated at 37°C for 30 minutes to allow colistin to elute. Then, 50 µL of bacterial inoculum was added to all tubes, including the control, using a micropipette. The tubes were gently vortexed for uniform mixing and incubated overnight at 37°C. The MIC was determined as the lowest concentration of colistin that visibly inhibited bacterial growth [Table/Fig-2] [11,12].



[Table/Fig-2]: Disc Elution (DE) test.

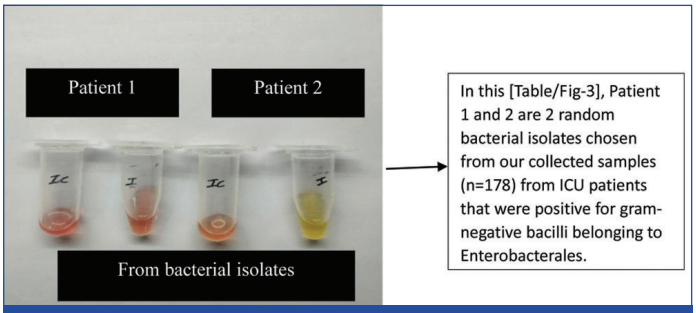
Rapid Colistin NP Test [13]: This test is a modification of Nordmann Poirel rapid colistin NP test [14]. The rapid colistin NP solution was prepared by mixing Cation-Adjusted Mueller-Hinton Broth (CAMHB) (6.25 g), phenol red (0.0125 g), and 225 mL of distilled water. The pH was adjusted to 6.7, and the solution was autoclaved at 121°C for 15 minutes. Once cooled to room temperature, 25 mL of anhydrous D-glucose was added, creating 250 mL of solution A.

solution B was prepared by combining solution A with colistin at a final concentration of 0.2 mg/mL.

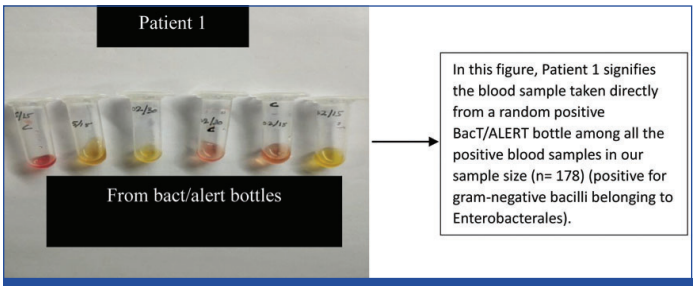
For testing, two sets of Eppendorf tubes were prepared, each containing two rows. In the first set, solution A (without colistin) was added to the first-row tubes as a control, while solution B (with colistin) was added to the second-row tubes. In the second set, solution A and solution B were similarly added.

- In the first set, 100 µL of incubated sub-cultured bacterial isolate (from culture plates) was added to each tube.
- In the second set, 100 µL of blood (from BacT/ALERT bottles flagged for GNB) was added directly.

All tubes were incubated at 37°C for four hours. A positive result was indicated by a clear colour change from red to yellow, while no colour change indicated resistance [Table/Fig-3,4] [15].



[Table/Fig-3]: Rapid Colistin NP test from bacterial isolates showing colour change from red to yellow denotes positive reaction. In the figure, "IC" stands for isolate control "I" stands for isolate.



[Table/Fig-4]: Rapid Colistin NP test performed from Bact/ALERT bottles showing positive reaction is denoted by colour change from red to yellow that signifies for colistin resistance.

STATISTICAL ANALYSIS

Statistical analysis was performed using IBM SPSS software. The Chi-square test was used to assess the correlation between BMD (the gold standard method) and other methods such as DE and the rapid colistin NP Test (from both isolates and BacT/ALERT bottles). A p-value of <0.05 was considered statistically significant.

RESULTS

In the present study, a varied isolation of GNB was obtained from blood samples. Among 178 bacterial isolates, 46%(81/178) *Escherichia coli*, 24.1%(43/178) *Klebsiella pneumoniae*, 1.6%(3/178) *Enterobacter cloacae*, 0.5%(1/178) *Klebsiella oxytoca*, 0.5%(1/178) *Citrobacter spp*, 6.7%(12/178) *Salmonella Typhi*, 4.4%(8/178) *Salmonella Paratyphi A*, 1.1%(2/178) *Salmonella Paratyphi B*, 0.5%(1/178) *Proteus mirabilis*, 7.8%(14/178) *Acinetobacter baumanii*, 1.6%(3/178) *Acinetobacter lwoffii*, 1.6%(3/178) *Morganella morganii*, 1.6%(3/178) *Providencia spp.*, 1.1%(2/178) *Serratia marcescens*, 0.5%(1/178) *Moraxella spp* were obtained.

In [Table/Fig-5-7], it is clearly shown that the total number of bacterial isolates included in this study was 178, all belonging to Enterobacterales. Two phenotypic methods-BMD and DE were performed to determine the MIC of colistin for these isolates. Additionally, antimicrobial susceptibility testing for colistin was conducted using the rapid colistin NP test, both from bacterial isolates and directly from BacT/ALERT bottles.

Broth Microdilution (BMD)		Disc Elution (DE) Sensitive	Disc Elution (DE) Resistant	Total
Sensitive	Count	151	0	151
	% within Broth Microdilution (BMD)	100.0%	0.0%	100.0%
Resistant	Count	0	27	27
	% within Broth Microdilution (BMD)	0.0%	100.0%	100.0%
Total	Count	151	27	178
	% within Broth Microdilution (BMD)	84.8%	15.2%	100.0%

Chi-square tests

	Value	df	p-value	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-square	178.000[a]	1	0.0001	Exact Sig. (2-sided): <0.001	Exact Sig. (1-sided): <0.001

[Table/Fig-5]: Chi-square results of Disc Elution (DE) test and Broth Microdilution (BMD).

Broth Microdilution (BMD)		Rapid Colistin NP test performed on bacterial isolates: Sensitive	Rapid Colistin NP test performed on bacterial isolates: Resistant	Total
Sensitive	Count	151	0	151
	% within Broth Microdilution (BMD)	100.0%	0.0%	100.0%
Resistant	Count	2	25	27
	% within Broth Microdilution (BMD)	7.4%	92.6%	100.0%
Total	Count	153	25	178
	% within Broth Microdilution (BMD)	86.0%	14.0%	100.0%

Chi-square tests

	Value	df	p-value	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-square	162.660[a]	1	0.0001	Exact Sig. (2-sided): <0.001	Exact Sig. (1-sided): <0.001

[Table/Fig-6]: Chi-square results of rapid colistin NP test performed on bacterial isolates.

			Rapid Colistin NP test directly from BacT/ALERT bottles: Sensitive	Rapid Colistin NP test directly from BacT/ALERT bottles: Resistant	Total
Broth Microdilution (BMD)	Sensitive	Count	151	0	151
		% within Broth Microdilution (BMD)	100.0%	0.0%	100.0%
	Resistant	Count	3	24	27
		% within Broth Microdilution (BMD)	11.1%	88.9%	100.0%
Total		Count	154	24	178
		% within Broth Microdilution (BMD)	86.5%	13.5%	100.0%

Chi-square tests

	Value	df	p-value	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-square	155.140[a]	1	0.000	Exact Sig. (2-sided): <0.001	Exact Sig. (1-sided): <0.001

[Table/Fig-7]: Chi-square test results of rapid colistin NP test directly from BacT/ALERT bottles.

When BMD was performed, 151 out of 178 isolates (84.8%) were found to be sensitive to colistin, while 27 out of 178 (15.2%) were resistant. The results for DE were identical. The rapid colistin NP test performed on bacterial isolates showed that 153 out of 178 (85.9%) were sensitive to colistin, and 25 out of 178 (14.0%) were resistant. Similarly, when the rapid colistin NP Test was conducted directly from BacT/ALERT bottles, 154 out of 178 isolates (86.5%) were sensitive to colistin, whereas 24 out of 178 (13.4%) were resistant. In this study, BMD was the gold standard method.

In [Table/Fig-8], it is clearly depicted that sensitivity and specificity for Rapid Colistin NP Test were 92.5% and 100%, respectively (for bacterial isolates) and 88.9% and 100% (for BacT/ALERT bottles). Similarly, PPV and NPV for rapid colistin NP Test were 100% and 98.6%, respectively (for bacterial isolates) and 100% and 98.1%, respectively (for BacT/ALERT bottles). Pearson's Chi-square test was used for the statistical analysis, wherein the correlation of BMD and DE, BMD and rapid colistin NP Test (from isolates and BacT/ALERT bottles) were made and the p-value came out to be significant ($p \leq 0.001$) for all the comparisons.

Parameters	Sensitivity	Specificity	Positive Predictive Value (PPV)	Negative Predictive Value (NPV)
Rapid Colistin NP test performed on bacterial isolates	92.5%	100%	100%	98.6%
Rapid Colistin NP tests performed directly on BacT/ ALERT bottles	88.9%	100%	100%	98.1%

[Table/Fig-8]: Sensitivity, specificity, PPV, NPV for rapid colistin NP test (from bacterial isolates and Bact/ALERT bottles).

DISCUSSION

Polymyxins are a group of antibiotics that work against GNB. They are known for their activity against MDR bacteria. *Paenibacillus polymyxa* is a bacterium known to produce polymyxins, including polymyxins A, B, C, D, and E. Polymyxin E is also commonly known as colistin [16]. It is used clinically, mainly against GNB like *Pseudomonas aeruginosa* and various Enterobacterales members, including MDR strains. Polymyxins act by disrupting the bacterial cell membrane, leading to cell death. Due to their potential for nephrotoxicity and neurotoxicity, they are often reserved for serious infections when other antibiotics have failed.

Colistin is available in two forms: Colistin Methanesulfonate (CMS) sodium for intravenous use and Colistin Sulfate (CS) for oral administration. It is considered one of the last lines of defense against MDR GNB infections, particularly within the Enterobacterales order [17]. The emergence of colistin resistance among bacteria has further emphasised the need for its prudent use to preserve its effectiveness as a treatment option.

In this study, (January 2023 to December 2023), 178 bacterial isolates belonging to the Enterobacterales order were analysed. All bacterial isolates were GNB. To determine the MIC for colistin, two phenotypic methods were performed: BMD and DE. Additionally, antimicrobial susceptibility testing for colistin was conducted using the Rapid Colistin NP Test, both from bacterial isolates and directly from BacT/ALERT bottles.

The first phenotypic method performed was BMD, the gold standard reference method for determining colistin MIC. Out of 178 bacterial isolates, 151 (84.8%) were sensitive, and 27 (15.2%) were resistant to colistin. In another study, conducted over five months (October 2016 to February 2017) in a hospital in Beirut, Lebanon, rectal specimens were collected from 23 ICU patients. These patients had received either carbapenem, colistin, or both during their ICU admission. Among the 23 bacterial isolates, 12 were found to be colistin-resistant by BMD [18].

The second phenotypic method used was DE for colistin MIC determination. Again, 178 bacterial isolates from Enterobacterales were tested, with 151 (84.8%) found to be sensitive and 27 (15.2%) resistant to colistin. The MIC for resistant strains was ≥4 µg/mL. A cross-sectional study conducted from March 2021 to April 2022 at a hospital in Ambala, India, included 857 bacterial isolates from Enterobacterales [19]. Colistin MIC was determined using DE, revealing that 806 (94.04%) isolates were sensitive and 51 (5.95%) were resistant, with resistant strains showing an MIC of ≥4 µg/mL.

For antimicrobial susceptibility testing in the present study, the rapid colistin NP Test was performed on bacterial isolates from Enterobacterales as well as directly from BacT/ALERT bottles. When tested from bacterial isolates, 153 (85.9%) were sensitive and 25 (14.0%) resistant to colistin. Lastly, in the present study, the rapid colistin NP Test was also performed directly from blood culture bottles flagged for GNB from Enterobacterales. This novel method involved standardising the test protocol by increasing the volume of blood sample added to peptone water (for dilution) from 0.1 mL to 0.2 mL [20]. Further incubation of the diluted blood sample was carried out for 30 minutes to allow for accurate interpretation. Using this method, 154 (86.51%) bacterial isolates were sensitive to colistin, while 24 (13.5%) were resistant. The sensitivity and specificity of this technique were 88.9% and 100%, respectively.

Limitation(s)

The limitations of the study included the small number of colistin-resistant GNB belonging to Enterobacterales. A follow-up multicentric study with a larger sample size, involving patients from various parts of the country, is imperative to confirm the results of the present study.

CONCLUSION(S)

The present study demonstrates that the rapid colistin NP test is an effective and reliable method for the early detection of colistin resistance in GNB within the Enterobacterales group. The test showed high sensitivity and specificity, offering rapid results that can significantly aid clinical decision-making. Its implementation can facilitate timely initiation of appropriate antimicrobial therapy, helping to curb the spread of resistant strains and improve patient outcomes. These findings support the routine use of the rapid colistin NP Test in clinical microbiology laboratories for the prompt identification of colistin-resistant pathogens.

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