Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/3wyj9h20

# KNOW POLYMYXINS BETTER TO COMBAT ANTIMICROBIAL RESISTANCE: MICROBROTH DILUTION METHOD RECOMMENDED ROUTINE TESTING METHOD FOR SCREENING COLISTIN RESISTANCE IN GRAM NEGATIVE BACTERIA:

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## ABSTRACT

Introduction: With increasing threat of multidrug-resistant organisms (MDROs), Colistin has become popular in clinical practice. Minimum inhibitory concentrations (MICs) are used to monitor Colistin resistance. Microbroth dilution (MBD) stays as the reference testing method for determination of resistance to Colistin and is considered essential for patient management.

Aim: To evaluate performance of MBD over E-strip and automated systems for the determination of Colistin MIC.

Methodology: Study conducted in Microbiology Department of tertiary care hospital of western Uttar Pradesh from March-September 2024. Total of non-repeat clinically significant strains of common Gram-negative bacteria (GNBs) isolated from various clinical specimen of patients received from various departments of hospital for routine Culture Sensitivity testing were processed. MIC was obtained by E-strip, MBD and automated system was interpreted and compared as per NCDC guidelines.

Results: Total 232 GNBs isolated from various clinical specimens were studied for in-vitro Colistin susceptibility by E- Strip method, MBD and BD Phoenix M-50 automated system. Majority of isolates tested were of *Escherichia coli* 132(55%), followed by *Pseudomonas aeruginosa* 42(17.5%), *Klebsiella spp.* 32(13.33%), *Acinetobacter baumannii complex* 26 (10.83%). The overall Colistin resistance found in 45(18.75%) isolates by gold standard BMD method. Categorical agreement (CA), Essential agreement (EA), Very major error (VME) and Major error (ME) rates for *Escherichia coli* were 84.85%, 57.14%, 3.78%, and 2.27% by Automated system and 87.88%, 77.67%, 1.51%, 4.54% by E-strip.

Conclusion: Different susceptibility testing methods for Colistin show great variation in their results and BMD may be considered as best choice over E- strip and automated method.

**Keywords*:*** Colistin, Antimicrobial susceptibility testing (AST), Minimum Inhibitory Concentration (MIC), E- Strip test, Microbroth dilution (MBD), BD Phoenix M-50 Automated system, NCDC (National Centres for disease control)

## INTRODUCTION

Living within an era of antibiotic resistance, Colistin, (cationic polypeptide) is an old antibiotic that regained popularity as a last resort drug to treat infections caused by Bacterial superbugs.[1]

The multi-drug Antimicrobial resistance among Gram-negative superbug bacteria has become a major public health issue impacting negatively on the clinical outcome of infected patients.[2]

The emerging resistance to Colistin (COL-R), whether arising by chromosomal mutations or by plasmid mediated (MCR) mechanisms is now recognized in humans, and it represents a new threat for global public health.[3]

Antimicrobial susceptibility testing (AST) for Colistin is a great challenge for a clinical laboratory because of multiple challenges faced in its testing, including less diffusion into Agar, inherent cationic properties of Colistin, the occurrence of hetero-resistance to Colistin in many species, and lack of a reliable reference method.[4]

A reliable and reproducible AST method is therefore required for patient management and for monitoring of Colistin resistance in multi drug resistant Gram-negative superbug bacteria.[5]

Because of several methodological issues associated with Minimum Inhibitory Concentration (MIC) testing of Colistin, both Clinical laboratory Standards Institute (CLSI) and National Centre for Disease control (NCDC), recommends use of Broth microdilution (BMD) method for susceptibility testing of colistin. Although, BMD method has not been adaptable for a clinical Microbiology laboratory because it is manual and quite labor intensive but other methods such as Epsilomer (E)strip method, Kirby- Beaur Disk diffusion (KBDD) method, and automated methods (Vitek-2, Phoenix, Microscan etc) though being less labour intensive and easy to perform but is associated with false susceptibility results when compared with BMD method.[6]

The studies pertaining to evaluation of other automated systems like Phoenix, Microscan and Sensi-titre systems with respect to BMD method for colistin susceptibility are scarce. This study was aimed to compare the results of Colistin susceptibility testing by three different methods mainly E-strip, BMD and BD Phoenix M-50 system with the reference Broth microdilution method to establish a practical and accurate approach for Colistin susceptibility testing in a Clinical Microbiology laboratory and to improve the patient outcome.

Emerging Colistin resistance in our area and selecting the accurate and cheaper method for reporting Colistin was challenge in our setup which prompted us to conduct this study.

To the best of our knowledge this is the first study from this part of the region.

## METHODOLOGY

**Setting:** This study was conducted over a period of six months (March to September 2024) in the Department of Microbiology, in a tertiary care Hospital of western Uttar Pradesh India.

A total of two hundred forty non-repeat clinically significant strains of common Gram-negative bacteria (GNBs) isolated from various clinical specimen of the patients received from various departments of the hospital for routine Culture Sensitivity testing were processed in the clinical Microbiology laboratory. The clinical specimens included Blood, Pus/Tissue, Body fluids, respiratory specimens and urine.

**Inclusion Criteria:** All the common GNBs (*Escherichia coli*, *Klebsiella* sp. *Acinetobacter baumanii* complex and *Pseudomonas aeruginosa*) obtained from the above specimens from both in-patient and out-patient who visited the hospital, of any age-group and gender received in the laboratory for Colistin susceptibility testing were included.

**Exclusion Criteria:** Isolates other than above mentioned GNBs and stool specimens were excluded from the study.

**Sample Processing**: Upon receipt in the Microbiology laboratory, all the clinical specimens were processed by the standard microbiological procedures. Three methods were used for Colistin testing that was E-strip, MBD and Automated BD Phoenix M-50 (Becton Dickinson, USA) system.

Disc Diffusion (Kirby Bauer) method is not the recommended method for Colistin testing by CLSI & NCDC, as MIC cannot be interpreted by this and higher false positive results due to less diffusion of Colistin in media.

The recommended method for Colistin testing is reporting of MIC value as per the standard CLSI guidelines. NCDC protocol was followed to conduct MBD procedure for Colistin testing.

In our study, Colistin testing by all the three methods was conducted for each of the common Gram-negative bacilli isolated from various clinical samples during the study period. The results from all the three methods were interpreted and compared. The procedure for three methods is described as below.

**A. Epsilomer (E) Strip Method:** A suspension of each isolate in Mueller-Hinton broth, adjusted to the density of a 0.5 McFarland standard, was swabbed in three directions to ensure uniform growth onto Mueller-Hinton agar plates. Once the agar surface was completely dry, an E-test colistin strip (ranging from 0.06 to 1,024 g/ml) was applied to each plate with sterile forceps, and the plates were incubated at 35°C for 16 to 20 h. The MIC was read where inhibition of growth intersected the E-test strip. When small colonies grew within the zone of inhibition or a haze of growth occurred around MIC end points, the highest MIC intersect was recorded.

**B. Microbroth Dilution Method (MBD):** It was done as per the latest NCDC guidelines, the Cation Adjusted Mueller-Hinton Broth (CaMHB) was prepared as per the manufacturer's instructions, primary stock solution of Colistin in concentration of 1000 µg/ ml was prepared by dissolving 10 mg of Colistin sulfate powder (Sigma; Potency=633 µg/ mg) in 6.33 ml of sterile water. It was aliquoted in smaller volumes and stored at -60 ºC. From the primary stock solution, working stock solution of 4x final concentration was made. Working stock of 64 µg/ ml was made by adding 64 µg/ ml from primary stock solution to 936 µg/ ml of autoclaved MHB broth. 500µl was added from the 64µg/ml working stock solution to 500µl MHB in Micro-centrifuge tube and twofold serial dilutions was performed to get drug concentrations as 32µg/ml, 16µg/ml, 8µg/ml, 4µg/ml and so on. Fresh dilutions of Colistin were made with every batch of test.

Preparation of 96 well-round bottom microtiter plate was done by adding 50µl of MHB broth to all wells of columns 1 to 10, 75µl in column 11, and 100µl in column 12 of the microtiter plate. Bacterial suspension was prepared in the concentration of 5 x $10^5$ CFU/ml and inoculated in micro-broth plate. The last two wells of each row of micro-broth plate acted as Growth control and Sterility control. Growth control well contained only adjusted bacterial suspension and Sterility control well contained only Cation adjusted Mueller Hinton Broth (CAMHB) which was used to prepare various dilutions.

In every batch Quality control strains are used as control. The controls used were *Escherichia coli* ATCC25922 (for testing Colistin against *Enterobacteriaceae*) and *Pseudomonas aeruginosa* ATCC27853 (for testing Colistin against *P. aeruginosa* and *Acinetobacter species*). The micro-broth plate was then incubated at 37ºC for 16-18 hrs. Reading of the test strains was taken only after satisfactory reading of the control strains. The minimum concentration of colistin which inhibits the visible growth of the bacteria was taken as its MIC. Interpretation of the results was done as per NCDC guidelines 2023. Based on epidemiological cut-off value for *Klebsiella sp*. and *Escherichia coli* were considered as sensitive if MIC value was ≤2µg/ml and as resistant if MIC value was ≥4 µg/ml. For *P. aeruginosa* and *Acinetobacter* sp. MIC value of ≤2 µg/ml was interpreted as sensitive and MIC value of ≥4 µg/ml was interpreted as resistant.

**C. BD Phoenix Automated System:** The manufacturer's instructions were followed to determine the colistin susceptibility of various test isolates. The probable range of MIC for BD Phoenix was ≤1 to >4µg/ml.

**Comparison between E-Strip versus BMD versus BD Phoenix results:** BMD was considered as gold standard for in-vitro susceptibility testing of Colistin and the MIC values obtained by BD

Phoenix was compared with E-strip and BMD. Essential Agreement (EA), Categorical Agreement (CA), Very Major Error rate (VME) and Major Error rates (ME) were calculated.

**Essential Agreement (EA)**: If the MIC of the isolates by BD Phoenix and E-strip were within +/- one doubling dilution in comparison to BMD, then the two methods were considered to be in essential agreement for that isolate.

**Categorical Agreement (CA)**: Those isolates which fall in the same category of interpretation were considered to be in categorical agreement for that isolate.

**Very-Major Error (VME)**: If the isolate was resistant by BMD and susceptible by other methods then it was considered as very major error (VME)

**Major Error (ME):** If the isolate was susceptible by BMD but resistant by other methods then it was considered as Major error (ME)

EA, CA, VME rate and ME rate were calculated as percentage. The International Organization for Standardization (ISO) established the following criteria for determining acceptable performance between the two methods: >90% for EA or CA and 3% for VME or ME. Re-confirmation of the discrepant results between BD phoenix and BMD methods (both VME and ME) was done by repeat testing.

## RESULTS

A total of 232 GNBs isolated from various clinical specimens were studied for in-vitro susceptibility for colistin by E-Strip method, Broth microdilution method and BD Phoenix M-50 automated system. Urine sample isolates accounts for 112 (48.27%), followed by blood 43(18.53%), pus 33(14.22%), Sputum 19 (8.19%), Sterile body fluids (bile, peritoneal fluid, pericardial fluid, ascitic fluid and CSF) 14 (6.03%), Endotracheal aspirates 8 (3.45%), Tissue culture isolates 3(1.3%). Overall, among 232 GNBs, tested the majority were of *Escherichia coli* 132(55%), followed by *Pseudomonas aeruginosa* 42(17.5%), *Klebsiella spp.* 32 (13.3%), *Acinetobacter baumannii complex* 26(10.8%). The overall resistance to Colistin amongst GNB (*Escherichia coli, Klebsiella* sp., *Acinetobacter baumannii complex* and *Pseudomonas aeruginosa*) was found to be in 45(18.75%) isolates by gold standard BMD method.

***Escherichia coli***: The total number of *Escherichia coli* strains which were sensitive to colistin by BD Phoenix method was 122 and by BMD were 108 and by E-strip 112. Eight strains were resistant by both BD Phoenix and BMD, showing similar results. Two strains resistant by BD Phoenix were found to be sensitive by BMD. Five strains which were sensitive to BD phoenix were found to be resistant by BMD. All the strains which were sensitive by BMD were also sensitive by E-strip. 4 isolates which were resistant by BMD were resistant to E-strips. Three isolates which were found to be resistant by E-strip were sensitive by BMD and also by BD Phoenix. Six strains which were sensitive by E-strip were found to be resistant by BMD and BD Phoenix.

***Klebsiella* sp**.: The total number of *Klebsiella* sp. strains which were sensitive to colistin by BD Phoenix method was 27 and by BMD were 24 and by E-strip 29. Out of 5 strains which were found to be resistant by BD Phoenix method were also found to be resistant by BMD method and 2 same isolates were also resistant by E-strip. Two strains which were sensitive by BD Phoenix, were resistant to BMD and 5 sensitive by E-strip were resistant by BMD and two by BD Phoenix. One isolate which was resistant by E-strip was found to be sensitive to BMD and by BD Phoenix.

***Acinetobacter sp***.: The total number of *Acinetobacter* strains which were found to be sensitive to colistin by BD Phoenix and BMD was 24 each and 25 by E-strip method. One strain was found to be resistant by all the three methods. Another strain which was found to be resistant by BD- Phoenix and BMD method was found to be sensitive by E-strip method. E-strips failed to detect resistance in isolates of *Acinetobacter* spp.

***Pseudomonas aeruginosa***: The total number of *Pseudomonas aeruginosa* strains which were found to be sensitive to colistin by BD-Phoenix method was 35 and by BMD method were 31 and 38 by E-strip. However, six strains detected resistant by BD-Phoenix method were also resistant by BMD and only 3 were found to be resistant by E-strip method showing similar results. Another three isolates

which were resistant to BD-Phoenix and BMD were sensitive by E-strip method. One isolate which was resistant to BMD and E-Strip method was found to be sensitive by BD Phoenix. One isolate was found to be only resistant by BD-Phoenix, was sensitive by BMD and E-strip. Four isolates were found to be only resistant by BMD but were found to be sensitive to E-strip and BD Phoenix, hence BD Phoenix and E strip failed to detect resistance in these four isolates of *Pseudomonas aeruginosa.* Comparative analysis for Colistin susceptibility testing for all bacterial isolates done by BD Phoenix, E-strip with the reference standard (BMD method) is summarized in Table 1.

**Table 1: Comparative Analysis between BD Phoenix, E-strip with the reference standard (BMD method) for Colistin susceptibility testing**

| **S.no.** | | **Categorical Agreement (CA)** | **Categorical Disagreement** | | |
|---|---|---|---|---|---|
| | | | | | |
| | | | **EA (%)** | **VME (%)** | **ME (%)** |
| **1.** | ***Escherichia coli* (132)** | | | | |
| | **BD/BMD** | 112 (84.85%) | 64(57.14%) | 5 (3.78%) | 3 (2.27%) |
| | **BMD/E-strip** | 116 (87.88%) | 87(77.67%) | 2(1.51%) | 6(4.54%) |
| **2.** | ***Klebsiella pneumoniae* (32)** | | | | |
| | **BD/BMD** | 27 (84.37%) | 14(51.85%) | 2(6.25%) | 0 |
| | **BMD/E-strip** | 26 (81.25%) | 19(73.07%) | 5(15.62%) | 1(3.12%) |
| **3.** | ***Pseudomonas aeruginosa* (42)** | | | | |
| | **BD/BMD** | 36(85.71%) | 20(4.76%) | 4(9.52%) | 1(2.38%) |
| | **BMD/E-strip** | 31(73.81%) | 27(64.28%) | 3(7.14%) | 1(2.38%) |
| **4.** | ***Acinetobacter* spp.** | | | | |
| | **BD/BMD** | 26(100%) | 21(80.77%) | 0 | 0 |
| | **BMD/E-strip** | 25(96.15%) | 14 (53.85%) | 1(3.85%) | 0 |

***EA- Essential agreement, CA- Categorical agreement, VME- Very Major Error, ME- Major Error**

## DISCUSSION

The emerging MDR in nosocomial GNBs has necessitated the use of Colistin among the few last therapeutic options for infections with these superbugs. Therefore, there is an increased need of accurate and reliable susceptibility testing methods for clinical laboratories worldwide to predict clinical response. Susceptibility testing for colistin is plagued with problems, such as the lack of consensus regarding breakpoints for resistance between the CLSI and EUCAST; the reported poor diffusion of colistin in the agar, the lack of correlation between different dilution methods, as well as lacunae in studies done on this group of antimicrobials, most of which have been done using colistin. In this study, we evaluated colistin MIC's obtained by three test methods (BD-Phoenix, BMD and E-strip) for the common GNBs isolated from various clinical samples at our hospital. MIC's obtained with BMD were used as the reference method. The rationale for evaluating these three different methods was to validate a system as accurate as BMD that would be more convenient for routine clinical laboratory use.

A discrepancy between two test methods (BD Phoenix versus BMD) was found to be 3.78%, 6.25%, 9.52% and 0% for *Escherichia coli, Klebsiella* sp., *Pseudomonas aeruginosa* and *Acinetobacter* sp. respectively. The discrepancy for *Pseudomonas aeruginosa* is apparently more because of the lower number of the isolates tested in the study. However, it is important to note that in *Pseudomonas aeruginosa* the isolates which were found to be colistin resistant by BMD, showed colistin sensitive

by BD Phoenix method. This is in contrast to *Klebsiella* sp., *Escherichia coli* and *Acinetobacter* sp. which were more detected as colistin resistant by BMD method than BD Phoenix method.
A discrepancy between two test methods (E-strip versus BMD) was found to be 4.54%, 15.62%, 7.14% and 3.85% for *Escherichia coli, Klebsiella* sp., *Pseudomonas aeruginosa* and *Acinetobacter* sp. respectively. The discrepancy for *Klebsiella* sp. is more due to variable reasons. Firstly, more diffusion of colistin into the agar, low media depth of the plate.
In our study, the categorical agreement of BD Phoenix for *Acinetobacter baumanii* complex (100%) was acceptable and that of *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* was marginally acceptable (84.85%, 84.37%, 85.71%) respectively. The categorical agreement of E-strip for *Acinetobacter baumanii* complex was acceptable (96.15%) and that of *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* was marginally acceptable (87.88%, 81.25%,73.81%) respectively. The Categorical and Essential disagreement in *Pseudomonas aeruginosa* was because of very major errors and not due to major errors. This disagreement in *Pseudomonas aeruginosa* can be attributed to the lesser number of *Pseudomonas aeruginosa* strains considered in our study.
In our study, for (Automated versus BMD) in *Acinetobacter baumannii* complex, EA/CA was found to be 80.77%/100% and VME /ME rates were 0%/0%.Vourli S *et al*. study found the EA/CA between Vitek-2 and BMD to be 88.9%/89.7% in *Acinetobacter baumanii* clinical isolates.[7]
However, for (E-strip versus BMD) *Acinetobacter baumannii* complex, EA/CA was found to be 53.85%/96.15% and VME /ME rates were 1%/0%. There was no ME with E-test while VME was 1%. This denotes it falsely identified one isolate of *Acinetobacter baumanni*complex as sensitive which was resistant by BMD. These values were in agreement to a study by Gupta *et al*.,[8] and Arroyo *et al*.[9]
Colistin gradient diffusion tests (E-tests and MIC strip tests) have also not been found to be suitable for the measurement of colistin MIC in clinical isolates in various studies. This could be attributed to different brands of E-strips used, thus hindering effective drug penetration through the agar medium. E-test is currently not recommended as a testing method for colistin MIC.

## CONCLUSION

There are no new antibiotics against MDR Gram-negative superbugs in the pipeline therefore Colistin, the last resort drug should be preserved and used judiciously after antibiotic susceptibility testing and following antibiotic stewardship. In our study, Colistin susceptibility testing was done simultaneously with BD-Phoenix, BMD and E-strip method and the agreement was marginally acceptable for *Escherichia coli, Klebsiella pneumoniae* and acceptable in *Acinetobacter baumannii* complex. In a tertiary health care facility, Colistin is used both empirically and therapeutically because of the type of patient population who are generally referred cases from primary/secondary health care facilities and already on high-end antibiotics or immune-supressed or post-transplant or malignancy patients who are on antimicrobial prophylaxis or treatment. In immunosuppressed or immunocompromised patients, Colistin susceptibility testing should be carried out and interpreted routinely using gold standard microbroth dilution method for deciding the optimum choice of drug for all the indicated organisms except *Pseudomonas aeruginosa* which requires further large scale testing.
Disk diffusion method should not be used as routine testing method for Colistin sensitivity as it gives most inconsistent results as compared to the reference standard method due to poor diffusibility of Colistin into the medium. E-test is less reliable for Colistin susceptibility due to considerably lower CA for Colistin. It failed to identify all resistant strains.

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