



Comparative evaluation of colistin broth disk elution test and broth micro dilution test among carbapenemase producing gram negative bacilli in all clinical isolates of a tertiary care hospital

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Abstract

Purpose: Increased Antimicrobial resistance among clinically important Gram-negative bacilli, especially Carbapenemase-producing Enterobacterales and Pseudomonas, has regained interest in Polymyxin E (Colistin) and Polymyxin B as a last resort of treatment. Broth micro-dilution (BMD) is Colistin's only recommended susceptibility testing, but this method is impractical for most clinical laboratories.

Aims & Objectives: The study aims to evaluate the accuracy of the Colistin Broth Disk Elution test (CBDE) and the Broth Micro Dilution (BMD) method in obtaining Colistin MIC for Carbapenem-resistant Gram-negative bacilli (CRGNB) among all clinical isolates.

Materials & Methods: A Cross-sectional descriptive study was conducted in the Department of Microbiology, NRIIMS, Vizag, from September 2022 to February 2023. During this period, 1496 clinical samples (including Blood, Purulent exudates, ET aspirates, and sterile body fluids) were collected by convenient sampling and processed. All Gram-negative bacilli isolates obtained from all age groups were included in our study. Repeated isolates from the same patient, isolates from stool samples and the organisms which are intrinsically resistant to Colistin, such as Proteus, Serratia, Providencia and Burkholderia species, were excluded from the study.

Methodology: Carbapenem-resistant Gram-negative bacilli which were detected through the Kirby Bauer disk diffusion method using Ertapenem & Meropenem discs and CLSI breakpoints, were selected and then subjected to Colistin BMD according to the Standard operating procedure of the National Programme on AMR Containment,

NCDC, India (August 2020) and CBDE test was conducted according to CLSI M100 S32 Performance standards. Results of CBDE were compared with BMD by using appropriate statistical tools.

Results: Among 1496 clinical samples processed, 108 Carbapenem-resistant isolates were identified, with *Klebsiella pneumoniae* (31%) being the most common. Of which 35.1% are from exudates, followed by 20.3% from blood, 16.6% from ET aspirates, 18.5% from Urine, 9.2% from BAL, and 5.5% from tissue samples. Most of the isolates (84) had an MIC ≤ 1 , while 18 had an MIC of 2 and 4 had an MIC of ≥ 4 in both methods, with 2 isolates showing discordant results. The Colistin Broth Disk Elution test (CBDE) showed a high level of agreement with the Broth Micro Dilution (BMD) method, with a categorical agreement (CA) of 98% and an essential agreement (EA) of 100%.

Conclusion: The study showed that the CBDE test can be used as an alternative to the BMD test for obtaining the MIC of Colistin, which is important for guiding the appropriate use of this last-resort antibiotic in clinical practice.

Keywords: Micro dilution, carbapenemase, bacilli, tertiary care hospital

Introduction

Increased Antimicrobial resistance among clinically important gram-negative bacilli has been a major global threat since last two decades and especially emergence of carbapenemase producing *Enterobacteriales*, *Pseudomonas* and *Acinetobacter* species has regained interest in polymyxins mainly polymyxin E (colistin) and polymyxin B as their last resort of treatment³.

But, unfortunately incidence of colistin resistance among them is also increasing now a days due to increased usage of this drug both in human and veterinary health care. As colistin is a large polycationic molecule which has poor diffusion capacity into agar thus, routinely performed disk diffusion or gradient diffusion tests cannot accurately detect colistin resistance⁴.

In 2015, both the Clinical and Laboratory Standard Institute (CLSI) and the European Committee of Antimicrobial Susceptibility Testing (EUCAST) joint working group has approved Broth Micro Dilution (BMD) as a reference method for antimicrobial susceptibility testing of colistin (6), but it cannot be routinely performed as it is a labor intensive procedure and difficulty to perform due to polysorbate effectiveness and binding of colistin to microtitre plates, these properties made invitro susceptibility testing of colistin cumbersome and challenging. Since 2020 CLSI has also approved Colistin Broth Disk Elution Test (CBDE) and Colistin Agar Test (CAT) for colistin susceptibility testing (20).

There is a need of standardizing colistin testing method which is accurate and feasible for all clinical laboratories to perform. The present study is mainly conducted to evaluate accuracy of CBDE test with that of BMD test in obtaining Minimum Inhibitory Concentration (MIC) of colistin.

Materials and Methods

A Cross sectional descriptive study was conducted in Microbiology Lab (Department of Microbiology NRIIMS, Visakhapatnam, South India). All Gram negative bacilli isolates which were obtained during the period of September 2022 to February 2023 from clinical samples of all age groups which were sent for routine diagnostic evaluation are included in our study and Repeated isolates from same patient, Isolates

from stool samples, Organisms which are intrinsically resistant to colistin like *Proteus*, *Serratia*, *Providencia* and *Burkholderia* species were excluded from the study.

Methodology

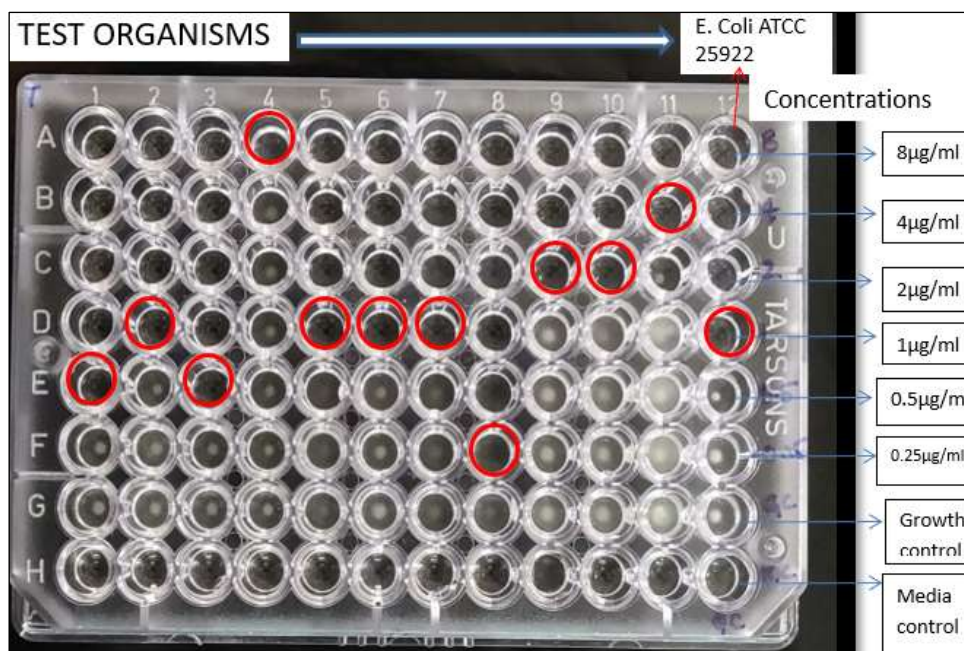
During the study period 1496 clinical samples (including Blood, Purulent exudates, ET aspirates, Sterile body fluids, Urine) were collected and processed in our clinical laboratory. Out of them 452 Gram negative bacilli isolates were obtained.

Among them 108(23.8%) Carbapenem resistant isolates were detected through Kirby Bauer Disk Diffusion method using 10µg Meropenem discs and CLSI breakpoints. All the isolates were subjected to colistin BMD according to Standard Operating Procedure of Programme on AMR containment, NCDC, India (August 2020). For this procedure, Cation adjusted Muller Hinton Broth (CAMHB) media and colistin sulphate powder acquired from Himedia Laboratories, India. Stock solution (1,000 µg/ml) prepared by adding 10mg of colistin sulphate powder in 6.33ml of autoclaved distilled water. We have prepared 4 times of Working stock solution to attain the required concentration of colistin in microtiter wells. Desired concentration of Working solution (0.25 to 8µg/ml) were made by two fold serial dilutions.

For making 32µg/ml (4 X Working solution)
from original stock solution of (1000µg/ml)
 $C_1V_1=C_2V_2$
1000µg/ml X V₁=32 µg/ml X 1000µl
V₁=32µl

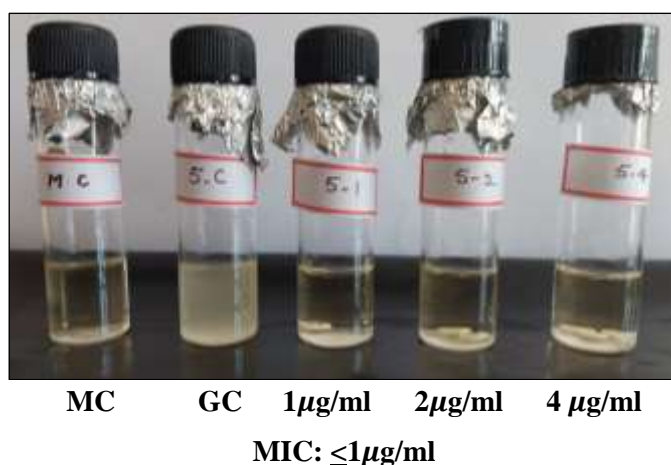
	500 µl					
	1	1:2(2)	1:2(3)	1:2(4)	1:2(5)	1:2(6)
MCT	968 sterile MHB +32µl working stock solution	500 sterile MHB	500 sterile MHB	500 sterile MHB	500 sterile MHB	500 sterile MHB
Colistin Conc. (µg/ml) in MCT	32	16	8	4	2	1
From MCT take 25µl and add to all wells in corresponding microtiter plate column containing 50µl CAMHB						
Final conc. (µg/ml) in microtitre plate wells	8	4	2	1	0.5	0.25

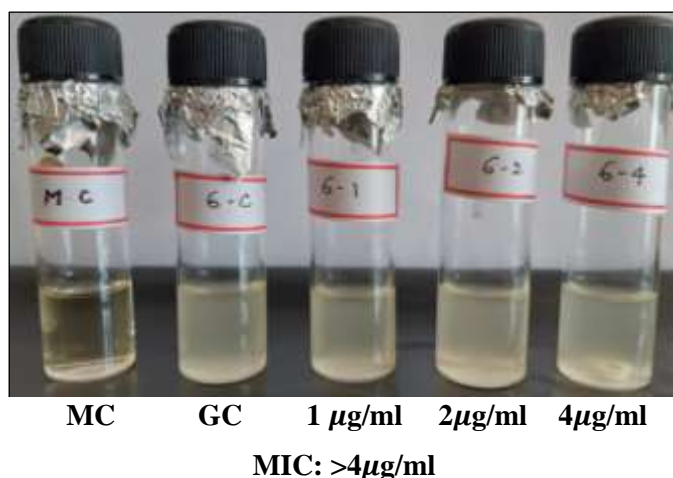
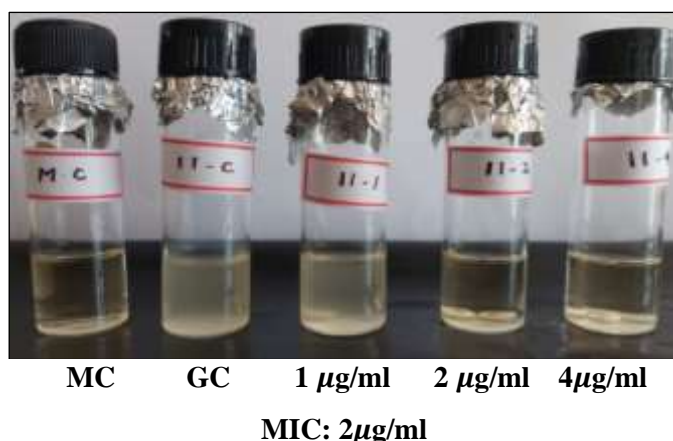
Standardized inoculum of 0.5 McFarland was prepared using Direct colony suspension method. This inoculum is diluted 1:75 times by adding 10µL to 740µL of autoclaved MHB medium to yield bacterial concentration of 5x10⁵ CFU/well. To achieve 100µL in each well of microtitre plate, test wells are added with 25µL of working solution, 25µL of inoculum and 50µL OF CAMHB ;75µL of CAMHB, 25µL of inoculum was added in Growth Control well and only 100µL CAMHB was added to Media Control well and incubated at 35 °C for 18 to 24hrs.



Colistin MIC's of test isolates in Broth Micro Dilution

CBDE test was performed according to CLSI 2020 M100 S32 performance standards. For this procedure CAMHB and 10µg colistin sulfate discs were procured from Himedia Laboratories, India. 10ml of CAMHB was filled in each tube and 4 tubes are labeled as control, 1, 2, 4µg/ml. 1, 2 and 4 disks were added into tubes labelled 1, 2 and 4µg/ml respectively. Then tubes are gently vortexed and had let the colistin elute from the disks for 30min to 1hour at room temperature. No disks were added to Growth control tube. 50µL of standardized inoculum of 0.5 McFarland added to each tube including Growth control tube and incubated at 35°C for 18 to 20hrs. For both methods, As Quality Control strains *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 and an In house Positive control strain were used.





Colistin Mic's of Test Isolates in CBDE

Interpretation

The lowest concentration of drug at which the visible growth is completely inhibited after incubation is considered as MIC. Results of BMD and CBDE were interpreted based on colistin MIC breakpoints as recommended by 2021 CLSI guidelines. MIC results of CBDE were compared with that of reference BMD test. Agreement between CBDE and BMD were described as Categorical Agreement (CA) and Essential Agreement (EA).

Results

Among 452 Gram negative bacilli isolates, 108(23.8%) carbapenem resistant isolates were identified with *Klebsiella pneumoniae* (34/108,31.4%) being most common then followed by *Acinetobacter baumannii* (28/108,25.9%), *Escherichia coli* (22/108,20.3%), *Pseudomonas aeruginosa* (20/108,18.5%), *Klebsiella oxytoca* (4/108,3.7%) as shown in (Table. No: 1)

As shown in Table.no:2, Majority of isolates were obtained from Purulent exudates (35.1%), Blood (22/108,20.3%), ET aspirate (18/108, 16.6%), Urine (18.5%), BAL fluid (10/108,9.2%), Tissue samples (6/108,5.5%).

Majority of the isolates were obtained from patient suffering with Surgical Site Infections (32/108,29.6%), Ventilator Associated Pneumonia (18/108,16.6%), Sepsis (11.1%), Pulmonary disease (10.4%), Chronic kidney disease (8.2%), Poly trauma (5.5%), others (18.5%).

Out of 108, 84 isolates (77.8%) had MIC $\leq 1\mu\text{g/ml}$, while 18 isolates (16.7%) had MIC of $2\mu\text{g/ml}$ and 4 isolates (3.7%) had MIC $\geq 4\mu\text{g/ml}$ in both methods, with 2 isolates (1.8%) showed MIC of 2 in test method and 4 in reference BMD. Out of 108 CRGNB, 6(5.5%) isolates showed resistance for colistin by BMD. Results in our study represents CBDE test has an EA of 100% and CA of 98.1%.

Table 1: Distribution of Study isolates

Organism	No. of isolates	Percentage
<i>Klebsiella pneumoniae</i>	34	31.4%
<i>Acinetobacter baumannii</i>	28	25.9%
<i>Pseudomonas aeruginosa</i>	20	18.5%
<i>Klebsiella oxytoca</i>	4	3.7%

Table 2: Sample wise distribution of CRGNB Isolate

Samples	Percentage of isolates obtained
Purulent exudates	35.1%
Blood	20.3%
E.T aspirates	16.6%
Urine	18.5%
BAL fluid samples	9.2%
Tissue samples	5.5%

Table 3: Distribution of Colistin MIC of CBDE with reference BMD

		MIC of BMD \longrightarrow					
MIC of CBDE \downarrow	MIC($\mu\text{g/ml}$)	0.25	0.5	1	2	4	8
	≤ 1	8	30	46	0	0	0
	2	0	0	0	18	2	0
	4	0	0	0	0	2	
	>4	0	0	0	0	0	2

*Interpretation: $\leq 2\mu\text{g/ml}$: Intermediate ; $\geq 4\mu\text{g/ml}$: resistant.



Minor error(mE)-MIC: $4\mu\text{g/ml}$ (BMD) and $2\mu\text{g/ml}$ (CBDE).

Table 4: Colistin MIC of CRGNB isolates in reference BMD

MIC	No. of isolates	Percentage
$\leq 1\mu\text{g/ml}$	84	77.7%
$2\mu\text{g/ml}$	18	16.6%
$\geq 4\mu\text{g/ml}$	6	5.5%

Table 5: Organism wise distribution of colistin MIC

Organism	No. of isolates tested	MIC of BMD					
		0.25	0.5	1	2	4	8
<i>Klebsiella pneumoniae</i>	34	0	4	16	10	2	2
<i>Acinetobacter baumannii</i>	28	2	12	8	4	2	0
<i>Escherichia coli</i>	22	4	4	12	2	0	0
<i>Pseudomonas aeruginosa</i>	20	0	8	10	2	0	0
<i>Klebsiella oxytoca</i>	4	2	2	0	0	0	0

Discussion

Carbapenem resistant Gram-negative bacilli has gained major importance in health care system as they are mainly associated with nosocomial infections and are difficult to treat. Polymyxins are considered as last choice of antibiotics for CRGNB infections. Especially colistin which is widely used in both human and veterinary health which lead to development of colistin resistance. And due to lack of a feasible test for colistin susceptibility testing, most of the laboratories cannot perform appropriate susceptibility testing for colistin.

This study is mainly conducted to assess the accuracy of CBDE test with that of reference colistin BMD. Prevalence of Carbapenem resistant GNB in the present study was 23.8%, which is correlated with Nair and vaz *et al.* [12] who reported 26%, K. Sreeja vamsi *et al.* [13] reported 38.3% in their respective studies.

In the present study, majority of isolates with carbapenem resistance was observed in patients of Surgical ICU and wards (35.1%), which is correlated with Namitha Thomas *et al.* [14] reported 36.2%, Dr. Elandevi *et al.* [15] reported 47.1%, Uddin mohammad *et al.* [16] reported 23%.

In the present study, Prevalence of colistin resistance among carbapenem resistant GNB in our study was 5.5%, which is correlated with Ayushi sharma *et al.* [17] reported 6.2%, Sujatha *et al.* [3] and Kar *et al.* [19] reported 11% and 14% of colistin resistance respectively.

In the present study, CA of CBDE test was 98% and EA was 100% with respect to reference BMD, which is correlated with Sujatha *et al.* [3] reported 98%, Swati sharma *et al.* [18] reported 98.4%, Simner *et al.* [7] reported 100% in their studies.

Conclusion

We here by conclude that our study showed a high degree of agreement between the two methods.

Therefore CBDE test can be used as an alternative to BMD test in obtaining MIC for colistin, which is important for guiding appropriate use of last resort antibiotic in clinical practice.

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