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Research article

Agar dilution test and its Merits and Drawbacks in evolving dynamics of colistin susceptibility

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ABSTRACT

Objectives: Automated results for determining colistin susceptibility tests are unreliable, micro broth dilution requires expertise, and CBDE has limited dilutions. International Consensus Document of 2022 suggested that agar dilution was unacceptable due to varying results in the literature.

Methods: The study was designed to evaluate the agar dilution method for colistin susceptibility in CRE isolates compared to CBDE. In the study, 108 carbapenem-resistant isolates were tested for Colistin susceptibility by Microbroth Dilution, agar dilution, and colistin broth disc elution. The comparisons were made using various statistical parameters.

Results: The results of the agar dilution method revealed an essential agreement of 75 % and a categorical agreement of 92.5 %. The method showed a sensitivity of 75 % and a specificity of 97.7 %. The positive and negative predictive values were 88.2 % and 94.5 %, respectively. Youden's index was 0.727, indicating a moderate level of accuracy. Meanwhile, CBDE diagnostic accuracy tests were better, with Youden's index at 0.939.

Conclusions: While CBDE demonstrated better accuracy parameters, it did not offer a broader MIC range. In contrast, agar dilution showed reasonable specificity and reliability for isolates with high MIC. Therefore, we propose using CBDE for screening and agar dilution as a supplementary test in the approach to colistin susceptibility.

1. Introduction

Colistin is often used for infections caused by carbapenem-resistant Enterobacterales when no other treatment options are available. However, testing for colistin susceptibility in microbiology labs can be difficult due to the molecule's structure and poor diffusibility. There are three approved colistin susceptibility methods for determining minimum inhibitory concentrations (MICs): Micro broth dilution (MBD), Agar dilution (AD), and Colistin broth disc elution (CBDE) [2]. Of these, Micro broth dilution is considered the gold standard. However, the recently formulated International Consensus Guidelines for the Optimal Use of Polymyxins [1]do not consider Agar dilution an acceptable susceptibility testing method due to varying results in the literature.

Agar dilution allows for testing multiple isolates and concentrations in a single plate, making it useful in resource-poor settings. Additionally, it can test a wider variety of organisms than Colistin Broth Disc Elution, which is only standardized for Enterobacterales

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and *Pseudomonas aeruginosa* [2]. This study was designed to evaluate the reliability of Agar dilution method in Carbapenem-resistant Enterobacterales (CRE) and compare it with other CLSI-approved methods.

2. Materials &methods

The study was conducted in the Microbiology Laboratory of AIIMS Patna from December 2021 to December 2022. A total of 108 non-repeating clinical carbapenem-resistant Enterobacterales clinical isolates were tested using colistin micro broth dilution, agar dilution, and colistin broth disc elution, following CLSI M 100 &M −07 [2,3] guidelines. Minimum inhibitory concentration (MIC) records were documented using electronic data management.

Microdilution assays were conducted against Colistin and Polymyxin B in untreated 96-well polystyrene microplates. U-bottomed microtiter plates without tween-80 were used for MIC determination [4]. Colistin sulphate salts were utilized in the assays. Dilutions ranging from 0.125 to 256 mg/l were prepared using cation-adjusted Muller Hinton broth. The minimum inhibitory concentrations (MICs) were observed after inoculation and incubation for 12–18 h under aerobic conditions. Agar dilution was performed according to CLSI-M07 [3], and Colistin broth disc elution was performed according to CLSI-M-100 [2].

Tests were done in duplicates with known resistance lab isolates, and ATCC *Escherichia coli* 25922 and *Pseudomonas aeruginosa* 27853 were put up with each batch to ensure accuracy.

EUCAST guidelines were relied upon for interpreting the susceptibility of Colistin as the Clinical and Laboratory Standards Institute (CLSI) does not offer specific interpretation criteria for defining susceptibility in this context. Colistin and Polymyxin B are considered equivalent agents [5], so the same interpretative criteria provided by EUCAST are applied to both molecules.

All the Colistin resistance isolates were screened for the mcr-1 gene using Hi-PCR Colistin resistance probe PCR Kit.[Product code: MBPCR209]

Statistical analyses were performed to assess the performance of the testing methods. Sensitivity, specificity, predictive values, and likelihood ratios were computed [13–17], with a particular emphasis on agar dilution compared to the gold standard, Microbroth Dilution [2]. The comparison of agar dilution and colistin broth disc elution MICs with Microbroth Dilution involved essential agreement, categorical agreement, Major error, and very major error determined as per predefined criteria [2,11].

3. Results

3.1. Description of isolates

Out of 108 Carbapenem-resistant Gram-negative bacilli tested.

The respiratory samples include Sputum and Endotracheal aspirates. The Pus samples include proper aspirated pus only, not swab samples. The sample-wise distribution of Organisms is listed in Table 1.

Most of the samples belonged to the ward (60/108*100 = 55.5 %) followed by ICUs (36/108*100 = 33.3 %), and a few samples were from OPD also (12/108*100 = 11.11 %).

3.1.1. MIC comparison of AD&CBDE

81.48 % were found to be sensitive by Micro-Broth Dilution, while 20 were found to be Colistin-resistant with a MIC ≥4. By agar dilution, 91 (84.25 %) were found to be sensitive, whereas in CBDE, among 108 isolates, 88 (68.75 %) were found to be sensitive using EUCAST interpretation guidelines [6], where MIC of ≤2 was considered sensitive. All the resistant isolates were negative for mcr1.

A scattergram was plotted to show all the 108 isolates' MIC from Agar dilution and Micro broth dilution for comparison of MIC by both methods (Fig. 1). The scattergram depicts the MIC comparison of AD & MBD, with the X-axis showing MICs of Colistin obtained from Agar dilution and the Y-axis showing MICs of Colistin obtained from Micro broth dilution. Six resistant isolates were missed in agar dilution, which is depicted in the scattergram in red boxes, and these are the "very major" errors. Two isolates were falsely given as resistant by agar dilution, indicated in orange, which is a "major error".

3.1.2. Diagnostic accuracy comparison

For the AD test, the Sensitivity is 75 %, Specificity is 97.9 %, PPV is 88.2 %, NPV is 94.5 %, PLR is 33, NLR is 0.256, and Youden's Index is 0.727. and For the CBDE test, the Sensitivity is 95 %, Specificity is 98.9 %, PPV is 95 %, NPV is 98.9 %, PLR is 83.6, NLR is

Table 1
Sample-wise distribution of Isolates.

Sample	Number of organisms	
	E-coli	Klebsiella pneumoniae
Pus	21	35
Respiratory samples	3	10
Urine	22	4
Blood	5	8

Respiratory samples include Sputum and Endotracheal aspirates.
Pus includes proper aspirated pus only, not swab samples.

AD →	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256
0.125	32	10	5	5			1	1				
0.25	6	7	3									
0.5	3		4	3	1							
1			1	2	1							
2	2		1									
4						3						
8	2							2				
16							1	1	2			
32	1						1		1			
64	1											
128	1					1					1	
256												2
MBD												

Fig. 1. Scattergram: Comparison of MICs of Microbroth Dilution & Agar Dilution.
Note: The X-axis denotes MIC values of Colistin, starting from 0.125 to 256, obtained from the Agar Dilution method. The Y-axis denotes MIC values of Colistin, starting from 0.125 to 256 obtained from Micro Broth Dilution. Red boxes indicate very major errors. The orange colour represents major errors. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

0.0506, and Youden's Index is 0.939.

The essential agreement on agar dilution was 75 %, and the categorical agreement was 92.5 %. Among errors, Major errors were 2.27 %, and very major errors were 15 %. Minor errors were not calculated as Colistin, according to EUCAST guidelines, has only sensitive and resistant breakpoints (no intermediate breakpoints) [6].Organism-wise distribution of agreements and errors of Agar dilution and Colistin, both disc elution, were given in Table no 2 and Table no 3, respectively.

3.2. Evaluation of agar dilution as a test for colistin sensitivity as per FDA guidelines for AST: organism-wise

Dilutions were charted organism group-wise. The x-axis includes the MIC of Micro broth dilution, and the Y-axis consists of the MIC of Agar dilution. Boxes represented by "ª" were excluded from calculating essential agreements since they are just the values at the end; exact values were not determined. (Figs. 2 and 3). Total evaluable isolates and Essential agreement among the Evaluable isolates were calculated from the scatter plot [9].

For *Klebsiella pneumoniae*, the overall essential agreement was found to be 72.2 %, while for evaluable isolates, it was found to be 75 % (Fig. 2).

For *Escherichia coli*, Overall essential agreement was found to be 80.76 & on evaluable isolates, it was 62.5 % (Fig. 3).

4. Discussion

The study thoroughly evaluated the recommended susceptibility testing of colistin in 108 carbapenem-resistant Enterobacterales, considering resource constraints and the unreliability of automated methods for colistin susceptibility [12]. The strategic exclusion of carbapenem-sensitive Enterobacterales strains from the study was based on the understanding that the likelihood of colistin administration in such strains is less. Unlike most previous studies that focused on essential agreement, categorical agreement, and errors, the current study calculated agreement on valuable isolates according to the FDA [9]. In addition, the study has incorporated sensitivity, specificity, and predictive values for a deeper level of comparison between the agar dilution and colistin broth disc elution [13–17].

While the agar dilution method offers several advantages, it also comes with significant limitations. Its potential for semi-automation of the testing process is beneficial in resource-limited laboratories. However, it's important to note that this method can be labour-intensive without automation, requiring significant time and effort. Additionally, the plates used for agar dilution are not readily available from commercial sources and must be prepared in-house. These plates have a limited shelf life and must be used within a week of preparation to ensure reliable and accurate results. This balanced view of the method's pros and cons is crucial for understanding its practicality and feasibility.

The colistin broth Disc Elution method, a relatively new approach, is highly adaptable and can be implemented in any laboratory, particularly those operating in resource-poor settings. This adaptability is a reassuring factor, confirming its applicability in various

Table 2
Agreement & Errors-Agar dilution -Organism Wise.

Organism	Essential agreement (EA)	Categorical agreement (CA)	Major error	Very major error
Klebsiella pneumoniae	39/54 * 100 = 72.22	49/54 * 100 = 90.74	2/88 * 100 = 2.27	3/20 * 100 = 15
E-coli	42/52 * 100 = 80.76	51/52 * 100 = 98.07	0	1/20 * 100 = 5
TOTAL	81/108*100 = 75	100/108*100 = 92.5	2/88*100 = 2.27	3/20*100 = 15 %

Note: Agreement & Errors are expressed as percentages. The total agreement is calculated for 108 isolates. EA AND CA should be >90 %, and the acceptable limit for MA and VMA is 3 % [2,7,8,11].

Table 3
Agreement & errors- colistin broth disc elution-organism wise.

Organism	Essential agreement (EA)	Categorical agreement (CA)	Major error	Very major error
Klebsiella pneumoniae	53/54 * 100 = 98.14	54/54 * 100 = 100	0	0
Escherichia coli	49/52 * 100 = 94.23	51/52 * 100 = 98.07	1/88 * 100 = 1.13	0
TOTAL	102/108*100 = 94.4 %	105/108*100 = 97.2 %	1/88*100 = 1.13	0

Note: Agreements and Errors were calculated as percentages. EA AND CA should be >90 %, and the acceptable limit for MA and VMA is 3 % [2,7,8,11].

AGAR DILUTION MIC	MICRO BROTH DILUTION MIC											
	0.125	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
0.125	11[a]	2[a]	1		1		2			1		[a]
0.25	4[a]	4										[a]
0.5	2[a]		4									[a]
1	4[a]		3	1								[a]
2	[a]		1	1								[a]
4	[a]					2						[a]
8	1[a]							1	1			[a]
16	1[a]						1	1				[a]
32	[a]							2	1			[a]
64	[a]											[a]
128	[a]											[a]
256	[a]									[a]	[a]	1[a]
Evaluable results		4	9	2	1	2	3	4	2	1		

Fig. 2. Distribution of MIC of colistin in Agar dilution vs Micro broth dilution, with respect to "evaluable result" as per FDA guideline for AST -*Klebsiella pneumoniae*(n-54).
Note: The scattergram shows "evaluable isolates" of *Klebsiella pneumoniae* when comparing Agar Dilution with Micro Broth Dilution. Values that fall within are excluded from the calculation. [Values with definite end points were only considered for the calculation of evaluable isolates; other MIC values were excluded.].

AGAR DILUTION	MICRO BROTH DILUTION MIC											
	0.125	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
0.125	21[a]	4[a]	2		1				1			[a]
0.25	6[a]	3										[a]
0.5	3[a]	3		1	1							[a]
1	1[a]			1								[a]
2	[a]											[a]
4	[a]					1					1	[a]
8	[a]											[a]
16	[a]						1					[a]
32	[a]											[a]
64	[a]											[a]
128	[a]											[a]
256	[a]									[a]	[a]	1[a]
Evaluable results		6	2	2	2	1	1		1		1	

Fig. 3. Distribution of MIC of colistin in Agar dilution vs Microbroth Dilution with respect to "evaluable result" as per FDA guideline [6] for AST -*Escherichia coli*(n = 52)
Note: The scattergram shows evaluable isolates of *E. coli* when comparing AD with MBD. [Values with definite end points were only considered for calculating evaluable isolates; other MIC values were excluded.].

settings. While this method does not allow for the calculation of "essential agreement on evaluable isolates" due to its limited dilution testing, it offers the significant advantage of requiring minimal materials and technical expertise, simplifying the testing process.

For agar dilution, an essential agreement was only 75 per cent, which is less than the accepted range [8,9]. Very major errors were 15 per cent, which is way beyond the acceptable 3 per cent [8,9]. Although the specificity and accuracy of the agar dilution are more

than 90 per cent, sensitivity was only 78.6 %. It can be inferred that agar dilution cannot be effectively used as a screening test. Moreover, Agar dilution has a higher Negative predictive value than a Positive predictive value. That means Agar dilution is a better test for detecting colistin-sensitive isolates. In other words, there is a probability that Agar dilution may miss some Colistin-resistant isolates. The essential and categorical agreement was more than 90 per cent for colistin broth disc elution. Major and very major errors were less than 3 per cent; both come in the acceptable range [8,9].

In a 2019 study by Kar et al. [11], the essential agreement of Agar dilution was 22 %, and the categorical agreement was 90 %. Major errors were 9 %, and very major errors were 10 %, which is on par with our study. According to Simpner et al. [7], for Colistin broth disc elution, EA and CA were 98 % and 99 % , respectively. Humphries et al. [8], EA and CA were 94 % and 97 %, respectively. All these results were concordant with the present study. To the best of our knowledge, none of the studies on colistin susceptibility testing had evaluated parameters such as sensitivity and specificity for agar dilution and colistin broth disc elution, so comparisons could not be made.

Further research is needed before generalising these findings to nonfermenters, as the present study only evaluated Enterobatereles**.** Colistin broth disc elution can be done only in Enterobactrales and *Pseudomonas aeruginosa* [2]; further studies are needed to find a better screening test for colistin resistance**.**

Our study evaluated all approved manual susceptibility methods for colistin, including agar dilution (AD), which has a lower sensitivity, making it unsuitable for screening for colistin resistance. Instead, we suggest using the colistin broth disc elution (CBDE) test for screening of Colistin resistance. However, AD can be used as a supplemental method to determine the exact minimum inhibitory concentration (MIC) of isolates and adjust the proper dose for colistin treatment, particularly for respiratory isolates, where the MIC should be less than 0.5. to achieve therapeutic efficacy [10].

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Ethical approval

Not applicable.

Data availability statement

Data have been deposited at Mendeley.
https://data.mendeley.com/datasets/zfzv4jdxkp/1.
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CRediT authorship contribution statement

Swathykrishna P.R: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Bhaskar Thakuria:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Formal analysis, Conceptualization. **Binod Kumar Pati:** Writing – review & editing, Visualization, Supervision, Methodology, Investigation, Formal analysis. **Prathyusha Kokkayil:** Writing – review & editing, Supervision, Software, Resources, Project administration, Investigation, Formal analysis. **Asim Sarfraz:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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