

COLISTIN AGAR; EVALUATION OF A NOVEL DIAGNOSTIC APPROACH TO DETECTION OF COLISTIN RESISTANCE

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ABSTRACT

Objective: To determine the diagnostic accuracy of Colistin agar for detection of Colistin resistance in clinical isolates of Multi-Drug Resistant Gram-Negative Bacilli.

Study Design: Cross-sectional validation study.

Place and Duration of Study: Department of Microbiology, Armed Forces Institute of Pathology, Rawalpindi Pakistan, from Feb to Aug 2019.

Methodology: A total of 100 Multi-Drug Resistant Gram-Negative Bacilli in clinical isolates were included. Isolates were identified using Gram stain, Catalase, Oxidase, API 20E, and API 20NE. After approval from the institutional ethical review committee, Colistin susceptibility was determined simultaneously by Colistin agar and Broth Micro Dilution Minimum Inhibitory Concentration method as per CLSI. For susceptibility criteria, EUCAST guidelines were followed. Results were validated with the gold standard test, i.e., Broth Micro Dilution.

Results: Out of 100 Multi-Drug Resistant clinical isolates, the distribution was *K. pneumoniae* n=60, *E.coli* n=16, *A. baumannii* n=11, *C. freundii* n=8, and *E. cloacae* n=5. The sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy of Colistin agar for detection of Colistin resistance, keeping Broth Micro Dilution Minimum Inhibitory Concentration method as the gold standard was 96.67%, 97.14%, 93.55%, 98.55%, and 97%, respectively.

Conclusion: Colistin agar has excellent diagnostic accuracy for the detection of colistin resistance with standardized inoculum density. Due to its ease of use, cost-effectiveness, and accurate results, it can be used in lab setups deficient in manpower and advanced equipment for Broth Micro Dilution or genetic sequencing.

Keywords: Agar Dilution, Broth Micro Dilution (BMD), Colistin Resistance, Gram-Negative Bacilli (GNB), Multi-Drug Resistant (MDR), Minimum inhibitory Concentration (MIC).

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INTRODUCTION

With the exponential rise in antibiotic resistance and paucity of research for newer antibiotics, the scales have tilted in favor of notorious pathogens creating havoc in healthcare setups.¹ Among MDR pathogens, MDR GNB (GNB) are the leading cause of nosocomial life-threatening infection with multi or even pan drug resistance.² Presently, physicians are reverting to Colistin as a routine last-line drug for the treatment of these infections. As is the case with all antibiotics, this exposure has started a rapidly rising trend of colistin resistance in MDR GNB, clearly indicating the dawn of post-antibiotic era.³ According to a recent study conducted in Karachi, Pakistan, colistin resistance was found in 15.9% carbapenem-resistant *Enterobacterales*.⁴

Currently, Broth Micro Dilution (BMD) is the gold standard method for detection of Minimum Inhibitory

Concentration (MIC). Still, it requires cation adjustment of broth in a narrow range as well as it is easily affected by adsorption to plastic. It is also time-consuming and has a subjective interpretation.⁵ Colistin Broth Disk Elution (CBDE) is also recommended for MIC detection but has limited detection in the lower range.⁶ Disk Diffusion is not recommended for susceptibility of Colistin as due to large molecular size diffusion in agar is variable.⁷

Agar dilution is also recommended for determining Colistin MICs in *Enterobacterales*. It was first described by Schmith and Reyman for the detection of MICs of sulphapyridine for gonococci.⁸ It has the advantage over broth dilution by the ease to perform with non-subjective interpretative criteria.⁹ Currently, for routine antimicrobial susceptibility, breakpoint methods are being used.¹⁰ Breakpoint methods rely on one or more cut off concentrations of antibiotic being tested, which categorize the test organism into susceptible or resistant.

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By amalgamation of agar dilution and breakpoint principles, we developed an in-house colistin agar with Muller Hinton agar base containing fixed Colistin concentration of 2ug/ml and cations adjusted as per CLSI guidelines. It was proposed to be used as a single inoculation for multiple isolates on the same plate divided into quadrants. The rationale of this study was to determine the diagnostic accuracy of Colistin agar for identification of Colistin resistant Gram-negative bacilli to help in prompt detection of Colistin resistance and preventing treatment failures in critical patients. To assess primarily ease of performance, cutting down the workload and cost-effectiveness, especially in resource-limited setups of labs.

METHODOLOGY

This cross-sectional validation study was carried out at the department of Microbiology, Armed Forces Institute of Pathology, Rawalpindi Pakistan, from February to August 2019. Permission of this study was obtained from Institutional Ethical Review Board vide FC-MIC18-13/READ-IRB/19/741. A sample size of 100 was calculated using a sensitivity specificity calculator, keeping prevalence of colistin resistance at 15.9%, estimated sensitivity and specificity at 96%, and confidence interval of 95%.^{11,6} Non-probability consecutive sampling was done.

To evaluate the diagnostic accuracy of the Colistin agar, non-duplicate 100 isolates of MDR Gram-negative bacilli were included having resistance to one agent of three or more antibiotic classes.¹² These isolates were obtained from clinical specimens, and identification was based on colony morphology, Gram stain, Catalase, Oxidase, API 20E, & API 20NE (BioMérieux).

Colistin MICs determination was carried out for these isolates by reference BMD method in Cation-Adjusted Mueller Hinton broth (CAMB) as recommended by Clinical Laboratory Standard Institute (CLSI), 2019 guidelines.^{13,14} Isolates with Colistin MICs ≤ 2 $\mu\text{g}/\text{mL}$ were categorized as sensitive and those with MICs > 2 $\mu\text{g}/\text{mL}$ as resistant as per EUCAST guidelines (2019).¹⁵ EUCAST guidelines were followed as interpretative criteria for colistin susceptibility was established in CLSI 2019. For BMD, 96 well microtiter plates were used. Each plate was vertically inoculated and consisted of 10 isolates with two controls. *E. coli* ATCC 25922 was used as positive (Colistin sensitive) control while for negative (Colistin resistant) control in house *K. pneumoniae* was used after confirming MIC of 8 $\mu\text{g}/\text{mL}$ by both BMD and ViTEK automated system due to unavailability of *E. coli* NCTC 13846. (Figure-1).

Each plate had sterility and growth controls as well. Colistin Sulfate powder (Sigma-Aldrich: Lot No: LRAA4721) was used to make an antimicrobial solution of 32 $\mu\text{g}/\text{mL}$ concentration, diluted two folds over six wells from 16 $\mu\text{g}/\text{mL}$ to 0.5 $\mu\text{g}/\text{mL}$. Each well was inoculated with 0.05 ml of antimicrobial agent except growth control well. Isolate inoculum was prepared to a final density of 5×10^5 CFU/ml, and 0.05 ml was dispensed in each well except sterility well. This inoculum was also applied on a quadrant of Blood agar and MacConkey agar to rule out any contamination during this procedure.



Figure-1: 96 well microtiter inoculated plate.

Colistin agar plates used in this study were based on the principle of agar dilution guidelines by CLSI. Foremost, the antimicrobial stock solution was prepared from the same batch of Colistin sulfate powder by Sigma-Aldrich. As per the manufacturer, this batch had a potency of 773 $\mu\text{g}/\text{mg}$. So as per formula, Weight (mg) = Vol (ml) \times Conc ($\mu\text{g}/\text{mL}$)/Potency ($\mu\text{g}/\text{mg}$), antimicrobial powder of 1.293 mg was used to make a stock solution of 50 ml having final concentration of 20 $\mu\text{g}/\text{mL}$. This solution was divided into 10 ml aliquots each and stored at -20 $^{\circ}\text{C}$ for future use. Muller Hinton (MH) agar base (Oxoid) was prepared as per manufacturer's instructions, and one vial of thawed antibiotic stock solution was diluted in 90 ml of molten MH agar for 1:10 dilution reaching a final concentration of Colistin per plate at 2 $\mu\text{g}/\text{mL}$. Prepared plates were stored at $2-8^{\circ}\text{C}$. These plates were allowed to warm at room temperature before inoculation. A 0.5 McFarland density inoculum was prepared of selected MDR isolates, and it was diluted to 1:10 in normal saline. This diluted inoculum was streaked onto agar plates divided into quadrants. The plates were incubated in aerobic conditions at 37°C for 18-24 hours. Any growth was considered as Colistin resistant isolate and no growth as Colistin sensitive isolate (Figure-2).

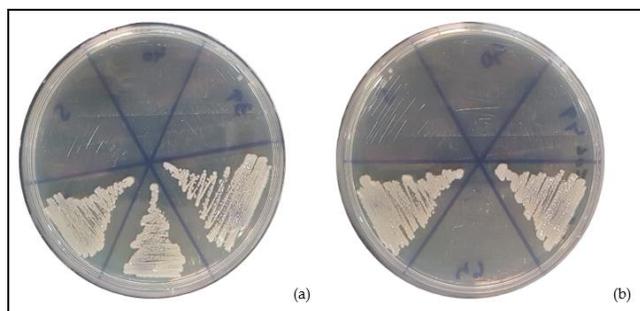


Figure-2(a,b): Colistin agar after 18-hour incubation at 35°C.

All the data collected was entered in SPSS version 25 and analyzed by applying correlations to ascertain significance. ROC curve was measured for sensitivity and specificity.

RESULTS

The distribution of 100 MDR GNB isolates in this study is shown in Figure-3. Among these isolates, Colistin resistance of 40%, 18.75%, and 27.27% was found in *K. pneumoniae*, *E. coli*, and *A. baumannii*, respectively. Isolates of *C. freundii* and *E. cloacae* were all sensitive to Colistin in this study.

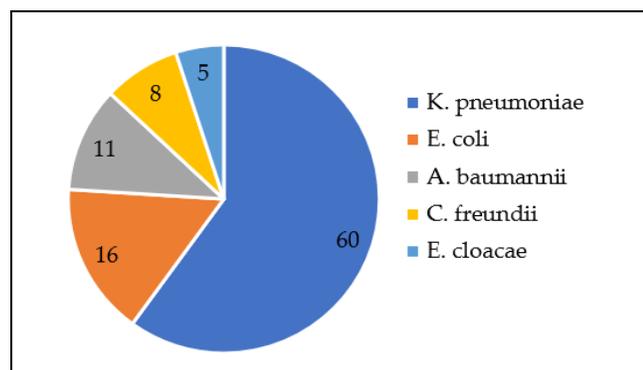


Figure-3: Distribution of clinical isolates.

All the isolates were subjected to the Colistin agar test, and 29 were found to be True Positive while two were False Positive. Among, Colistin agar sensitive isolates, one (False Negative) had Colistin resistance on the BMD method, whereas 46 (True Negative) had no Colistin resistance on the BMD method ($p=0.0001$) as shown in Table-I.

Table-I: Diagnostic accuracy of Colistin Agar.

| | Broth Microdilution MIC ≥ 4 $\mu\text{g/mL}$ | Broth Microdilution MIC ≤ 2 $\mu\text{g/mL}$ |
|-------------------------|---|---|
| Colistin agar resistant | 29 (TP) | 2 (FP) |
| Colistin agar sensitive | 1 (FN) | 68 (TN) |

TP=True positive, FP=False positive, FN=False negative, TN=True negative.

The diagnostic parameters of Colistin agar, for detection of Colistin resistance in clinical isolates of multidrug-resistant Gram-negative bacilli, keeping BMD method as the gold standard is shown in Table-II.

Table-II: Diagnostic parameters of Colistin Agar.

| S. No. | Variable | Result |
|--------|---------------------------|--------|
| 1 | Sensitivity | 96.67% |
| 2 | Specificity | 97.14% |
| 3 | Positive Predictive Value | 93.55% |
| 4 | Negative Predictive Value | 98.55% |
| 5 | Diagnostic Accuracy | 97% |

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DISCUSSION

Considering the steep rise in resistance against Colistin, there is an imperative need for a method to detect it in minimal time with high accuracy to treat morbidity caused by these bacteria as well as ensure proper measures to reduce their spread.¹⁶ Colistin agar has the potential to overcome these criteria with the added benefit of cost-effectiveness as on a single plate, up to 10 isolates can be tested simultaneously. Since this study is novel, there is no similar study available for comparison, both nationally and internationally.

In a study conducted by Humphries *et al*, for evaluation of colistin agar dilution test, there was 99.7% categorical agreement for detection of colistin resistance in *Enterobacteriales* with only 0.5% very major errors and no major errors.¹⁷

The Colistin broth disk elution method is also recommended for colistin MICs and has sensitivity and specificity comparable to our test.⁶ However, it requires elution of antibiotic discs in broth, which is susceptible to multiple factors, requires a larger volume of reagents hence costly, and relies on turbidity as growth indicator rendering result analysis subjective.

Rapid Polymyxin NP is a phenotypic method for detection of Colistin resistance in *Enterobacteriales* with a shorter turnaround time of 2-4 hrs as its only advantage. Rapid polymyxin NP requires a higher level of expertise.¹⁸ The sensitivity of Rapid polymyxin NP is higher at 98.1%, but specificity was lower at 94.9% than Colistin agar in our study.¹⁹

Chromogenic media are also available for the detection of colistin resistance by various manufacturers. They rely on a similar principle of agar dilution, but the exact amount of antibiotic is not disclosed hence lack technical transparency. Various studies have evaluated these agars, and results have been quite variable.²⁰⁻²² Furthermore, they have two main drawbacks

of being expensive and utilization as screening test rather than diagnostic test.

A limitation of this study was the lack of comparison with molecular detection of the colistin resistance mechanism. MCR-1 confers low-level resistance, and by keeping colistin concentration of 2ug/ml, this agar can identify these isolates as well, but it will need more research.

CONCLUSION

The diagnostic accuracy of Colistin agar is excellent for detection of Colistin resistance in clinical isolates of multi-drug resistant Gram-negative bacilli. It can be used both for screening by direct application of specimen and as diagnostic by inoculating standardized density of organism.

RECOMMENDATION

It is recommended that Colistin agar should be used routinely as a prime modality for detection of Colistin resistance for early treatment and infection control in peripheral/small labs where BMD and genetic sequencing is not available, as well as large setups with a high workload.

Conflict of Interest: None.

Author's Contribution

SS: Data collection, analysis, interpretation, discussion of literature review, WH: Review of article & correction, FA: Discussion review & correction, RKA: Data analysis, results and discussion of literature, IAM: Data analysis & results, MS: Discussion & literature review.

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