Colistin, the last resort antibiotic: challenges in the implementation of its routine susceptibility testing

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Summary

Background: colistin has become a critical antibiotic for life-threatening multidrug resistance Gram-negative infections, particularly carbapenemase-producing bacteria. Detecting colistin resistance in routine microbiology laboratories is crucial for combating these fatal infections poses a challenge. Especially in developing countries, there is a need for a cost-effective, rapid, and user-friendly diagnostic method.

Objective: implementing the various available methods for colistin testing is a significant challenge in resource-limited settings due to logistic difficulties and the need for technical expertise.

Materials and Methods: this study shares experiences and insights gained while implementing *in-vitro* colistin susceptibility testing in a high-load bacteriology laboratory of a tertiary care center in Delhi, India. The following test methods for colistin susceptibility testing were incorporated in the routine antimicrobial susceptibility testing of our laboratory: Colistin Agar Test, Colistin Broth Disk Elution Test, Broth Microdilution susceptibility testing.

Results: inconsistent growth patterns were observed in the colistin agar dilution Minimum Inhibitory Concentration (MIC) method, which could be resolved only after the preparation of fresh plates containing that specific concentration of colistin. The contamination issue of plates on use over a few days was addressed by pouring agar containing various concentrations of colistin in cotton-plugged glass tubes. In the colistin broth disk elution test, due to the non-availability of screw-capped 10 mL glass tubes, MacCornety bottles (30 mL) were used. Subcultures were performed from the turbid wells to rule out the growth of contaminants when encountering discordant MIC values or skipped wells on the colistin broth microdilution test.

Conclusions: despite several technical issues in *in-vitro* colistin susceptibility testing, we have successfully implemented it in our laboratory. Our experiences can offer guidance to laboratories that are still in the process of implementing it.

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Introduction

According to the most recent Clinical Laboratory Standard Institute (CLSI) recommendations, colistin Broth Microdilution (BMD), Colistin Agar Test (CAT), and Colistin Broth Disc Elution (CBDE) methods are acceptable for determining colistin susceptibility for the *Enterobacterales* and *Pseudomonas aeruginosa* [14]. Colistin, a cationic, cyclic polypeptide antibiotic first introduced in Japan in 1947 from the soil bacterium *Paenibacillus polymyxa*, was approved for clinical use in 1959 [7]. Its use was discontinued in the 1980s due to nephrotoxicity and neurotoxicity concerns [11]. Colistin, also known as polymyxin E, has become a last-resort antibiotic in the present era and could be considered for the treatment of severe infections caused by Multidrug-Resistant (MDR) Gram-negative organisms [25]. The increased use of colistin due to the lack of novel antibiotics has necessitated the development of appropriate and rapid *in-vitro* antimicrobial susceptibility testing methods to facilitate proper management of the MDR Gram-negative pathogens [26].

The disc diffusion test, which is widely used in clinical laboratories, produced high error rates when compared to Minimum

Inhibitory Concentration (MIC)-based methods and is regarded as unreliable for detecting colistin resistance [1]. *In-vitro* colistin susceptibility testing is difficult because the cationic property of colistin influences adherence to the microtiter plate. Furthermore, electrostatic interactions of colistin with acid or sulphate groups result in poor diffusion of colistin into agar, resulting in smaller inhibition zones [12]. Commercially available forms of colistin include colistin sulphate and sodium Colistin Methane-Sulfonate (CMS). CMS is an inactive prodrug that hydrolyzes to form active colistin [2]. The joint European Committee on Antimicrobial Susceptibility Testing (EUCAST) and CLSI polymyxin breakpoint working group do not recommend disk diffusion and gradient diffusion tests for colistin susceptibility testing due to the problems associated with colistin as mentioned earlier [1].

However, implementing the various available methods for colistin testing is a significant challenge in resource-limited settings due to logistic difficulties and the need for technical expertise. The primary goal of our study was to identify the inherent challenges in incorporating routine colistin susceptibility testing and the suitable corrective measures required to achieve accurate, consistent, and reliable results for this critical last-resort antibiotic in a high-volume tertiary care center.

Materials and Methods

This study shares the experiences and insights gained while implementing *in-vitro* colistin susceptibility testing in a high-load bacteriology laboratory of a tertiary care center in Delhi, India. The following test methods for colistin susceptibility testing were incorporated into the routine antimicrobial susceptibility testing of our laboratory. All three methods were used to assess the susceptibility of 80 multi-drug resistant Gram-negative isolates to colistin. For quality control, *MCR-1*-positive *Escherichia coli* ATCC BAA-3170, *Escherichia coli* NCTC-13846 and *Pseudomonas aeruginosa* ATCC 27853 strains were used as positive control. For negative control, *Escherichia coli* ATCC 25922 was used with each test.

Colistin Agar Test for Enterobacterales and *Pseudomonas aeruginosa*

The CAT is an agar dilution technique used to determine in vitro colistin susceptibility. It is a quantitative method used to test *Enterobacterales* and *Pseudomonas aeruginosa* in clinical microbiology laboratories. Cation-Adjusted Mueller-Hinton Broth (CAMHB) (Himedia Laboratories; Mumbai, India) is the medium used for agar dilution susceptibility testing of *Enterobacterales* and *Pseudomonas aeruginosa*. After checking the pH of each batch, fresh Mueller-Hinton Agar (MHA) were prepared on the day the colistin dilutions were added to agar plates (7.2 to 7.4) [4,14].

Potency calculation in our laboratory

The potency of colistin powder was determined by comparing it to the pure agent of colistin, which has a potency of 30,000 UN/mg, or 30 UN/g. The potency of colistin powder, which is available in colistin sulphate salt in our laboratory, is 19000 UN/mg.

Then potency with reference to pure agent =19000 UN/mg ÷ 30000 UN/mg =0.633 UN/mg or 633.33 UN/µg.

The primary stock solution was prepared by adding 10 mg of colistin sulphate powder with a potency of 633.3 g/mg to 6.33 mL of autoclaved distilled water. The final concentration of active colistin sulphate in the primary stock solution (as calculated against the referenced pure salt) was 1 mg/mL. The primary stock solutions were stored in sterile 1.5-2 mL cryovials at -70°C.

Preparation of Mueller Hinton Agar plates containing different concentrations of colistin

Four flasks were used to prepare different dilutions of colistin agar plates, namely 4 g/mL, 2 g/mL, and 1 g/mL, with the fourth flask containing no colistin or 0 g/mL colistin. One hundred mL MHA was prepared and poured into each flask before autoclaving at 121°C for 15 minutes.

The following formula was used to prepare different concentrations of colistin-containing agar plates from a primary stock solution containing 1mg/ml of colistin sulphate: C1 V1 = C2 V2.

Table 1 depicts the calculation for the volumes of colistin that are to be added to the MHA containing different concentrations of colistin. For preparing colistin agar plates, 400 µL of 1 mg/mL primary stock solution of colistin was added to the molten 100 mL MHA flask that had been equilibrated in a water bath to 45 to 50°C. The final solution was finally poured on MHA plate labeled as 4 µg/mL. Likewise, for preparing 2 µg/mL, and 1 µg/mL colistin agar plates, 200 µL and 100 µL of 1 mg/mL colistin stock solution was added to the molten 100 mL MHA flask, and finally to agar plates (labelled as 2 µg/mL and 1 µg/mL). No antibiotic solution was added for the preparation of 0 µg/mL colistin agar plates. The agar plates were allowed to solidify at room temperature. The plates were stored at 2-8°C and used within 5-7 days.

Inoculum preparation and inoculation

Three to five well-isolated colonies of test/Quality Control (QC) strains were transferred to sterile saline (4-5 mL) to make a homogenous suspension. The turbidity of the inoculum was standardized to match 0.5 McFarland standard, which equals approximately $1.5x10^8$ Colony-Forming Unit (CFU)/mL. The prepared inoculum was further diluted to 1:10 in sterile saline. Each colistin agar plate of different concentrations was divided into 10-15 parts. Ten µL of the 1:10 dilution of each test strain was streaked into each part of the agar plate, which was incubated at 33-35°C for 16-20 hours. Quality control strains were put with every colistin agar plate. Figure 1 shows an inoculated plate of CAT.

Colistin Broth Disk Elution test for Enterobacterales and *Pseudomonas aeruginosa*

CBDE test works on the principle that antimicrobial discs of a known concentration were eluted in a predetermined volume of broth to obtain standard doubling dilutions to determine MICs. The

Table 1. Calculation for the volumes of colistin that are to be added to the Mueller-Hinton Agar (MHA) containing different concentrations of colistin.

For 4 µg/mL	For 2 µg/mL	For 1 µg/mL
C1 V1=C2 V21000 µg/mL V1= 4 µg/mL x 100 mL	C1 V1=C2 V21000 µg/mL V1= 2 µg/mL x 100 mL	C1 V1=C2 V21000 µg/mL V1= 1 µg/mL x 100 mL
V1=0.4 mL or 400 µL	V1=0.2 mL or 200 µL	V1=0.1 mL or 100 µL

CBDE method was performed using CA-MHB. Four tubes containing 10 mL each of CA-MHB were taken for each isolate. To the CA-MHB tubes, 0, 1, 2, and 4 colistin discs (10 g) (Becton, Dickinson & Co.; Sparks, MD, USA) were added to provide final concentrations of 0 (growth control), 1, 2, and 4 g/mL, respectively. To let the colistin elute from the disks properly, the tubes were vortexed and allowed to stand at room temperature for 30 minutes. Standardized inoculum was prepared by using suspending 3-5 colonies from a fresh (18-24 hours) agar plate to 4-5 mL sterile saline. The turbidity was standardized to match that of a McFarland 0.5 standard. A 50 µL aliquot of this standardized inoculum was added to each tube to attain a final inoculum concentration of approximately 7.5×10^5 CFU/mL. After 16-20 hours of incubation at 35°C, MIC values were read as the lowest concentration that completely inhibits the growth of the test isolate. Interpretation was done using CLSI breakpoints of ≤2 µg/mL as intermediate and ≥4 µg/mL as resistant for *Enterobacterales* and *P. aeruginosa*. Figure 2 shows the CBDE test bottles containing final microbial concentrations of 0 µg/mL (growth control), 1 µg/mL, 2 µg/mL and 4 µg/mL [4,24].

Broth microdilution susceptibility testing for Enterobacterales, Pseudomonas aeruginosa and *Acinetobacter* spp.

This method is used to determine the *in-vitro* susceptibility testing to colistin for *Enterobacterales*, *Pseudomonas aeruginosa,* and *Acinetobacter species*. The medium, cation-adjusted Mueller-Hinton broth, was the same as that used in the CBDE. The preparation of the drug stock solution and the determination of potency were also similar to those for CBDE. A working solution of colistin was prepared from the primary stock solution by making the final drug concentration four times. To achieve a final concentration of 16 µg/mL, a working stock solution of 64 µg/mL was prepared in a sterile Microcentrifuge Tube (MCT). For this, 64 µL from the primary stock solution was added to 936 µL of autoclaved MHB medium in another MCT [4,7].

Preparation of dilutions of colistin

Five hundred µL from the 64 µg/mL working stock solution was added to 500 µL MHB medium in MCT, and twofold serial dilutions (in 9 MCTs containing 500 µL MHB) were prepared to get drug concentrations as 32 µg/mL, 16 µg/mL, 8 µg/mL, 4 µg/mL and so on.

Preparation of 96 well-round bottom microtiter plates

For the addition of dilutions of colistin, 50 µL of MHB was added to all wells of columns 1 to 10, 75 µL in column 11, and 100 µL in column 12 of the microtiter plate. Twenty-five µL of colistin dilution 64 µg/mL was added to column 1, 32 µg/mL to column 2 and so on till 0.125 µg/mL in column 10 of the microtiter plate. Column 11 was used as growth control containing only media and bacterial inoculum while column 12 was media control containing

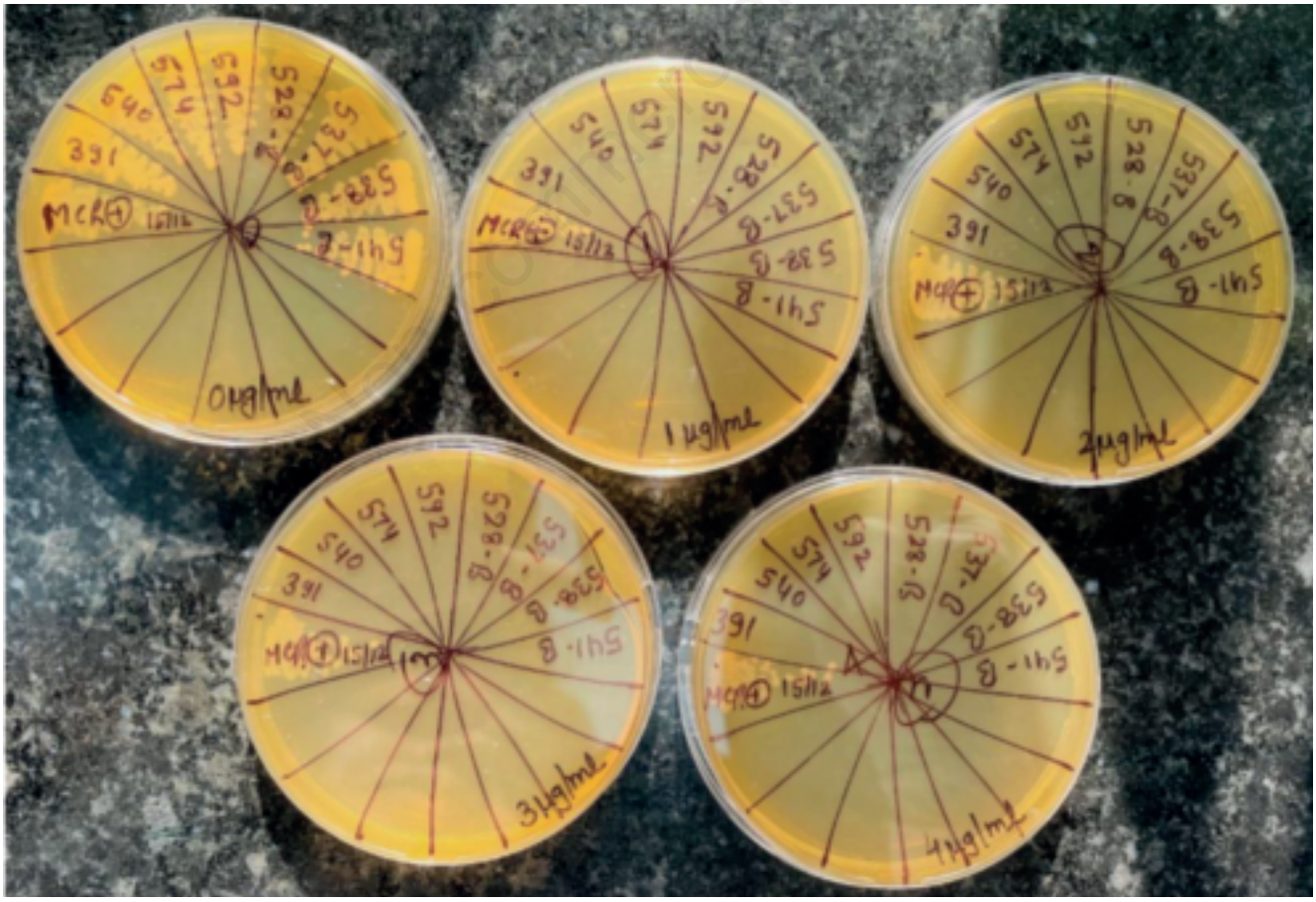

Figure 1. Inoculated plate of colistin agar test with positive quality control (QC) strain containing final colistin concentrations of 0 µg/mL (growth control), 1 µg/mL, 2 µg/mL, 3µg/mL and 4 µg/mL.

only media 100 µL. The final volume for each well of the microtiter plate was 100µL. Standardized inoculum of 0.5 McFarland was prepared and further diluted to 1:75 times by adding 10 µL to 740 µL of autoclaved MHB medium. From this diluted suspension, 25 µL was added to each of the wells in columns 1 to 11, already containing 75 µL (50 µL MHB + 25 µL antibiotic), to yield a bacterial concentration of approximately 5 x10^4 CFU/well. The microtiter plates were incubated at 35±2°C. MIC readings were taken after 16-18 hours of incubation as the lowest concentration of colistin completely inhibits the growth of the organism in the microdilution wells as detected by the unaided eye. Subcultures were performed from the turbid wells to rule out the growth of contaminants when encountering discordant MIC values or skipped wells.

Results

Practical hurdles faced and corrective actions implemented during in-vitro colistin susceptibility

Colistin agar dilution Minimum Inhibitory Concentration method

During standardization of colistin agar dilution MIC method for *Enterobacterales* and *Pseudomonas aeruginosa*, inconsistent growth pattern showing no growth in 2 µg/mL concentration agar plate but growth at 1µg/mL and 4 µg/mL was observed with positive QC Strain (*Escherichia coli* (NCTC13846) and *Escherichia coli* ATCC BAA-3170). On a colistin agar plate with a concentration of 0 µg/mL, the negative control (*Escherichia coli* ATCC 25922) showed growth, while no growth was noted at concentrations of 1 µg/mL, 2 µg/mL and 4 µg/m. *Pseudomonas aeruginosa* ATCC 27853 showed growth within the expected ranges as published by CLSI (*i.e.* MIC 1-4 µg/mL) at concentrations of 0 µg/mL, 1 µg/mL, and 4 µg/mL.

On a subsequent day, when two test MDR *Enterobacterales* isolates were tested along with repeat testing of positive QC strain on the same colistin agar test plates, identical inconsistent growth patterns were again registered. Outcomes remained the same even with the fresh 2 µg/mL colistin agar dilution test plate that was prepared simultaneously with the previous batch. After rechecking of calculations, a new batch of MHA plates containing 2 µg/mL concentration of colistin was prepared. Positive QC strain and two MDR *Enterobacterales* isolates were inoculated on fresh plates of 0,1,4 µg/mL and a new batch of 2 µg/mL colistin concentration plate. After incubation at 33-35°C for 16-20 hours, confluent growth was documented for positive QC strain in all four plates with differential colistin concentration. Colistin MIC for two MDR *Enterobacterales* isolates was ≤2 µg/mL.

In the colistin agar dilution MIC method, each set of colistin agar plates consisting of different concentrations (0,1,2 and 4 µg/mL) of colistin can determine the MIC of colistin of up to 10 bacterial isolates, including the positive QC control. We used the same set of plates over a duration till a total of 10 bacterial isolates of *Enterobacterales* or *Pseudomonas aeruginosa* were tested. Despite the best of our efforts, we struggled with instances of contamination on these plates with differential colistin concentrations on the use of the same set of plates over multiple occasions. In search of a viable solution, we decided to pour 2-3 mL of the differential colistin concentrations of 0,1,2 and 4 µg/mL in sterile glass test tubes of 12×100 mm with 7 mL capacity in a slant. Different colored dyes were used to mark the cotton plugs of glass tubes containing media with different concentrations of colistin poured into glass tubes (Figure 3). Each test bacterial isolate was inoculated as per standard guidelines on one set of tubes containing differential colistin concentration. With each batch of test isolates, a positive QC strain was tested. We documented similar results as the colistin agar dilution MIC method with our in-house modified test, and as no-repeat use of the same media was needed in this method, the issue of contamination was addressed effectively.

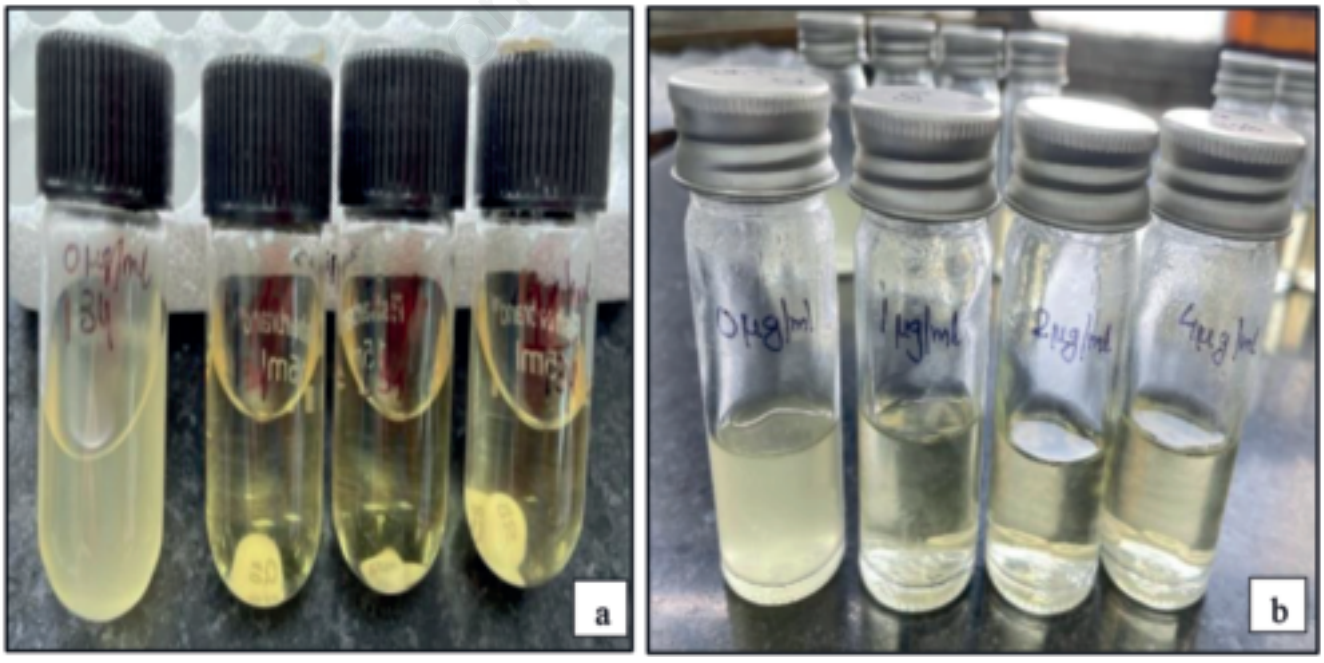

Figure 2. Colistin broth disk elution (CBDE) test bottles containing final colistin concentrations of 0 µg/mL (growth control), 1 µg/mL, 2 µg/mL and 4 µg/mL. a) Round bottom, 15 mL, screw-capped glass tubes for CBDE test; b) MacCornety, 30 mL, screw-capped bottles for CBDE test.

Colistin Broth Disk Elution test

The primary issue we faced in initiating CBDE was the non-availability of screw-capped 10 mL glass tubes. The procedure of CBDE involved vortexing, the available glass test tubes without screw-cap lids could not be used as the soggy cotton plugs may have resulted in sub-optimal testing conditions. We used 15 mL wide round bottom screw-capped glass tubes (Fisherbrand, Fisher Scientific; Hampton, USA) available to us (Figure 2). The results obtained with this modification to the standard technique were within range for positive QC strain. The issue we faced with these wide, round bottom tubes was to keep them in an erect position. Finally, widely available sterile screw-capped MacCornety bottles, 30 mL (Biochrom, Harvard Bioscience; Holliston, USA), were utilized in this test. The previous issues were addressed with this modification, as the MacCornety bottles are flat-bottomed, crew-capped glass containers that can be easily vortexed and can be kept in an erect position. The test results obtained were within range for positive QC strain.

Broth microdilution susceptibility testing

Arrangements for storing primary stock solutions of colistin had to be predefined as these cannot be stored in commonly available refrigerators. The temperature should not exceed -20°C, and self-defrosting freezers/refrigerators were not recommended.

On encountering discordant MIC values or skipped wells on BMD test, subcultures were performed from the turbid wells to rule out the growth of contaminants (Figure 4). Growth of gram-positive pathogens was observed in a few instances. Therefore, before considering other probable reasons for skipped wells, exclusion of contamination is advisable. Microtiter plates used initially for BMD testing were not provided with a cover. Plastic seals were used to cover the microtiter plates during incubation. Though we observed contamination in wells in rare instances, we switched to microtiter plates with ready-made covers to address this issue.

Discussion

Each recommended method for colistin susceptibility testing has its own set of challenges, as elaborated earlier; in addition, a few issues are inherent to all the methods involved. The purity of test strains is a prerequisite for accurate colistin susceptibility results. We ensured the proper subculturing of test strains and picking of only isolated colonies for inoculum preparation. Gram-positive pathogens, being intrinsically resistant to colistin, can grow well at various concentrations of colistin. This may lead to an erroneous interpretation of MIC values. With each batch of colistin susceptibility testing, the inclusion of routine QC strains is crucial. The success of each batch of testing is determined by the expected MIC QC range of these strains. Positive QC strain on subsequent subcultures demonstrated a decline in MIC values for colistin. Therefore, stock

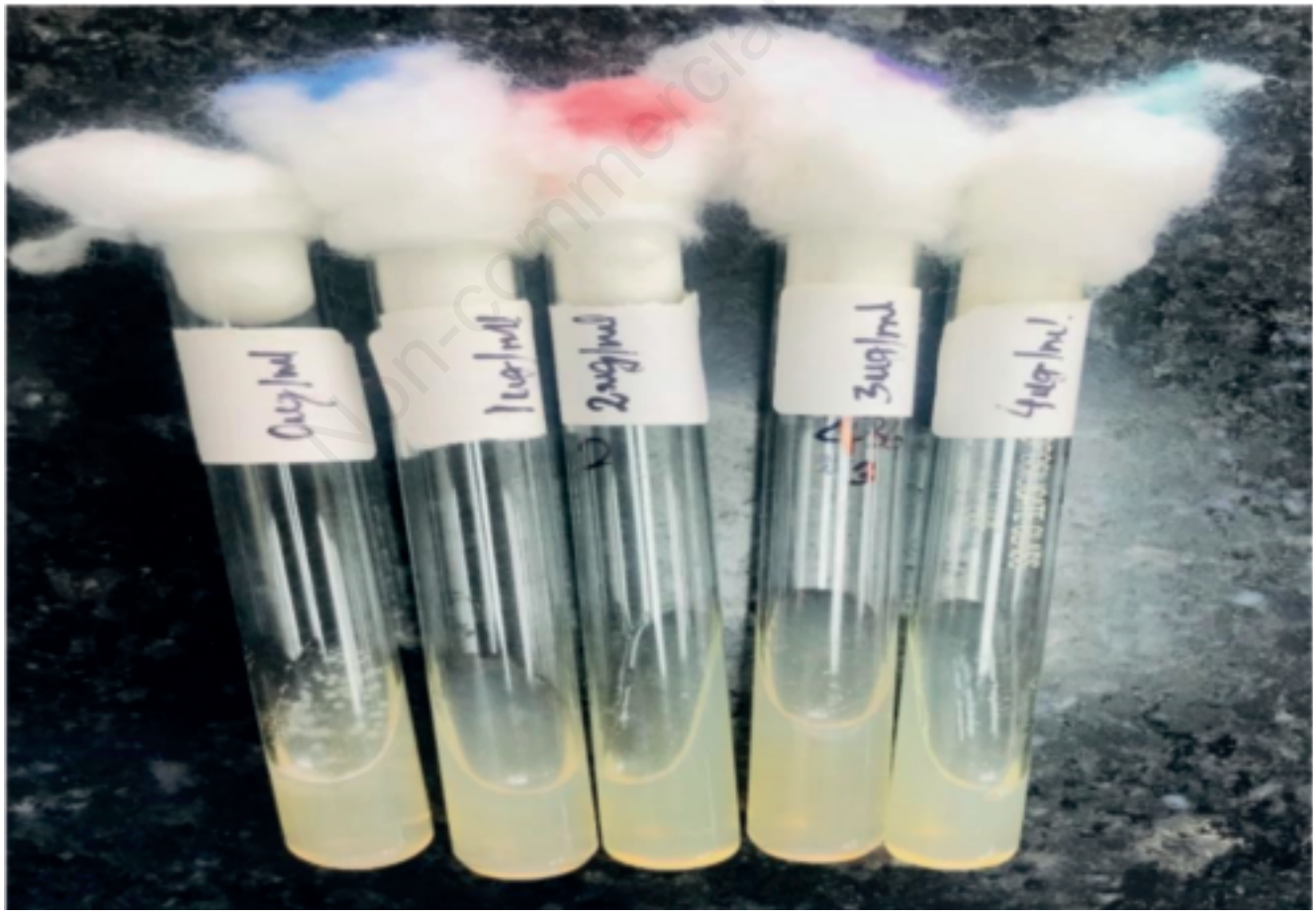

Figure 3. Inoculated tubes of colistin agar test with positive QC strain containing final colistin concentrations of 0 µg/mL (growth control), 1 µg/mL, 2 µg/mL, 3µg/mL and 4 µg/mL.

vials of QC strains had to be stored at -60°C and each week, new stock was revived and used for QC testing.

Any MIC determination method requires the proper interpretation of skip wells or tubes. CLSI guidelines state that a single skip well has no impact on the MIC reading [4]. However, test results showing multiple skip wells are seen as being uninterpretable. In our study, we encountered single skip wells at a lower concentration where adsorption was greater, so polymyxin adsorption to the polystyrene trays probably did not account for these results. According to a study by Landman *et al.* on polymyxin B MICs for *Enterobacter cloacae* and *Enterobacter aerogenes*, isolates with consistently indeterminate MICs exhibit higher levels of hetero-resistance, including at 64 g/mL. They concluded that critical concentrations propel the mechanisms leading to polymyxin resistance since the percentage of resistant subpopulations actually increased at concentrations of 2-8 g/mL of polymyxin B. This is consistent with the regular observation of skip wells at lower concentrations and the appearance of growth at concentrations of around 4 g/mL [18]. Napier *et al.* also reported Colistin-Hetero-Resistant Strain (colR/S) of *Enterobacter cloacae* from a bronchoalveolar lavage specimen from a kidney transplant patient [21]. Microbiologists often experience hetero-resistance while testing polymyxin susceptibility, especially in *Enterobacter, Acinetobacter,* or *Klebsiella spp*. [3,6,10,20,15]. The skipped-well isolates of *Pseudomonas aeruginosa* have been observed to have enhanced expression of pmrAB, phoQ, and the arn operon, according to a study on polymyxin B adaptation and inducible resistance in non-cystic fibrosis clinical isolates of *P. aeruginosa* [18,23]. However, the extent to which polymyxin B interpretations can be percolated to colistin is still to be determined.

Another issue with colistin is its affinity for adhering to plastic. The loss of colistin is up to 80% in dilution steps when fresh plastic comes in contact with the colistin solution [17]. The use of plastic microtiter plates could result in underestimated colistin concentrations within the wells. This situation could be mistaken for skipped wells where the concentration of the colistin in the well is accurate, but the pathogen exhibits enhanced expression of other drug resistance mechanisms. The results obtained by this method may be prone to inaccuracy. In such instances, it is advisable to subculture from skipped wells to check for the hetero-resistance of the bacteria. Additionally, it is recommended that the test be repeated with stringent quality control measures and freshly prepared colistin solutions to ascertain the MIC.

CBDE, on the other hand, is a relatively easy method and can be used to screen MDR gram-negative bacterial isolates for colistin resistance. Fenwick *et al.* used the CBDE and EDTA- CBDE methods to screen for plasmid-mediated colistin resistance among *Enterobacterales* and *P. aeruginosa* isolates directly from culture. Using the EDTA-CBDE method, they detected multiple mcr variants (*MCR* 1-4) and observed the sensitivity and specific of 100% and 94.3%, respectively [8]. Humphries *et al*. compared CBDE and CAT with reference BMD method using two inoculum volumes, 1 µL (CAT-1) and 10 µL (CAT-10) across 270 isolates of *Enterobacterales*, 122, *Pseudomonas aeruginosa*, and 106 *Acinetobacter spp*. They observed Categorical Agreement (CA) of 97.9% CBDE and BMD. They observed that the performance was better with CAT-10 over CAT-1 with CA of 94.9% (CAT-1) and CA of 98.3% (CAT-10) with reference BMD [13]. Both of these techniques exhibit low complexities in terms of performance and com-

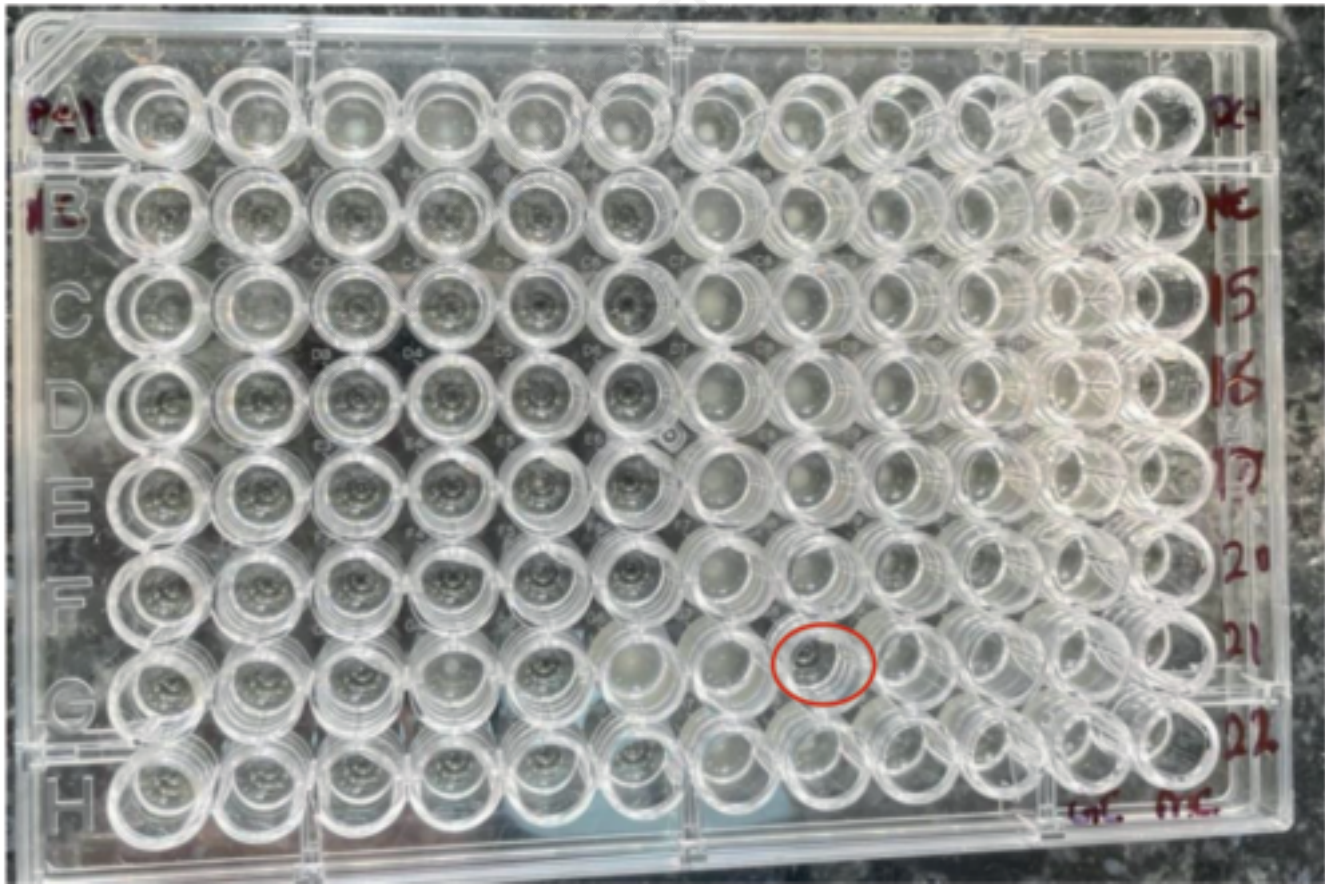

Figure 4. Colistin microbroth susceptibility plate showing skipped well.

Table 2. The advantages and disadvantages of all three methods for colistin susceptibility testing (n=80).

Method	Colistin broth disk elution test	Colistin agar test	Broth microdilution
Advantages	• Convenient to prepare the reagents • Simple and easy to perform • Can use commercially available colistin disks • Suitable for routine laboratory • Provides quantitative assessment of MIC • Economical in terms of cost	• Simple and easy to execute • Enables testing of multiple isolates on one plate • Identification of contaminant grown on solid media is easy • Provides an objective interpretation	• Gold Standard Method • Allows testing of multiple isolates on a single microtiter plate • Offers a broader range of MIC concentrations for colistin testing • Provides elaborate quantitative determination of MIC
Disadvantages	• In liquid media, distinguishing contaminant from the pathogen is challenging • Less accurate in comparison to the standard/reference method • Requires four tubes per isolate, leading to higher media consumption • Allows testing of fixed concentrations of colistin for MIC (0 µg/mL, 1 µg/mL, 2 µg/mL, 4 µg/mL) • Interpretation is subjective	• Plates may dry out • Risk of plate contamination when used for many days • Limited to testing of fixed concentrations of colistin for MIC (0 µg/mL, 1 µg/mL, 2 µg/mL, 4 µg/mL)	• Demands technical expertise for media preparation and testing • Potential for skipped wells or contamination • Probable reduced colistin concentrations with plastic plates microtiter

MIC, inimum inhibitory concentration.

parable yield as compared to the reference BMD method. Additionally, these approaches can be utilized for screening or in combination with established reference methods in case of hetero-resistance or skipped wells, thereby minimizing the chances of error. Table 2 summarizes the advantages and disadvantages of all three methods for colistin susceptibility testing.

Colistin susceptibility testing remains challenging for microbiology laboratories. Traditional methods like disk diffusion, gradient diffusion, and automated methods such as Vitek2 and Phoenix are not recommended, rendering much of the available data inaccurate [27]. Polymerase Chain Reaction (PCR)-based methods necessitate optimization and skilled technicians, although they are capable of identifying the few known resistance genes but may lag behind in detecting newly emerging *MCR* genes. Furthermore, the absence of *MCR* genes does not necessarily mean susceptibility, thereby preventing such isolates from being formally recognized on susceptibility assays [16,22].

New approaches, such as Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry, are being utilized to analyze the lipid A of bacteria. Colistin resistance in most Gram-negative bacteria stems from modifications to the lipid A portion of their Lipopolysaccharide (LPS), either through chromosomally encoded mutations or the activity of *MCR* proteins. Research has demonstrated that MALDI-TOF mass spectrometry can detect and characterize lipid A structures from various Gram-negative bacterial species directly from cultured bacterial colonies [16,19]. Additionally, the MALDIxin test has been developed to identify colistin resistance in isolated *Escherichia coli* colonies [5]. Another novel approach using flow cytometry enables rapid determination of AST within 2 hours, significantly reducing the time in comparison to the traditional two-day period. This can revolutionize diagnostic practices by allowing AST directly from positive blood cultures or colonies [9]. These innovative approaches may not be economically feasible and suitable for resource-limited settings, thereby leaving us to rely on simple and easy-to-perform tests such as CBDE, CAT, *etc.*

With the emergence of multidrug-resistant gram-negative pathogens, the susceptibility testing for colistin by all microbiology laboratories has become the need of this hour. Despite the easy availability of standard guidelines for various methods of recommended susceptibility testing, challenges often discourage the inclusion of colistin susceptibility testing in routine antibiotic susceptibility testing. This research that presents the firsthand account of a tertiary care hospital laboratory may prove to be very useful for the wider section of labs worldwide that are still in the process of developing their expertise for various recommended colistin susceptibility testing methods.

Conclusions

In the current scenario of high MDR prevalence, not providing colistin susceptibility is not an option. In numerous clinical situations, colistin is the only resort to antibiotic therapy. Despite available guidelines, many laboratories find colistin susceptibility testing to be a formidable challenge. Sharing the experiences and lessons learned from overcoming challenges in one laboratory can provide valuable insights for others, facilitating a smoother implementation of colistin susceptibility testing.

References

1. Bakthavatchalam YD, Veeraraghavan B. Challenges, issues and warnings from CLSI and EUCAST working group on polymyxin susceptibility testing. Journal of Clinical and Diagnostic Research: JCDR. 2017;11:DL03.
2. Bergen PJ, Li J, Rayner CR, Nation RL. Colistin methanesulfonate is an inactive prodrug of colistin against Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2006;50:1953-8.
3. Chew KL, La MV, Lin RT, Teo JW. Colistin and polymyxin B susceptibility testing for carbapenem-resistant and MCR-positive Enterobacteriaceae: comparison of Sensititre, MicroScan, Vitek 2, and Etest with broth microdilution. Journal of Clinical Microbiology. 2017;55:2609-16.
4. CLSI. Performance Standards for Antimicrobial Susceptibility Testing; Thirty Second edition. CLSI document M100-A32 edition. Wayne, PA: Clinical and Laboratory Standards Institute; 2022.
5. Dortet L, Bonnin RA, Pennisi I, et al. Rapid detection and discrimination of chromosome- and MCR-plasmid-mediated resistance to polymyxins by MALDI-TOF MS in Escherichia coli: the

MALDIxin test. J Antimicrob Chemother. 2018;73:3359-67.
6. Elias R, Melo-Cristino J, Lito L, et al. Klebsiella pneumoniae and colistin susceptibility testing: performance evaluation for broth microdilution, agar dilution and Minimum Inhibitory Concentration test strips and impact of the "skipped well" phenomenon. Diagnostics. 2021;11:2352.
7. Falagas ME, Kasiakou SK, Saravolatz LD. Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. Clinical Infectious Diseases. 2005;40:1333-41.
8. Fenwick AJ, Bergman Y, Lewis S, et al. Evaluation of the NG-Test MCR-1 lateral flow assay and EDTA-colistin broth disk elution methods to detect plasmid-mediated colistin resistance among Gram-negative bacterial isolates. Journal of Clinical Microbiology. 2020;58:10-128.
9. Fonseca e Silva D, Silva-Dias A, Gomes R, et al. Evaluation of rapid colistin susceptibility directly from positive blood cultures using a flow cytometry assay. Int. J. Antimicrob. Agents 2019;54:820-3.
10. Guérin F, Isnard C, Sinel C, et al. Cluster-dependent colistin hetero-resistance in Enterobacter cloacae complex. Journal of Antimicrobial Chemotherapy. 2016;71:3058-61.
11. Gupta S, Govil D, Kakar PN, et al. Colistin and polymyxin B: a re-emergence. Indian J Critical Care Med. 2009;13:49.
12. Humphries RM. Susceptibility testing of the polymyxins: where are we now? Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy. 2015;35:22-7.
13. Humphries RM, Green DA, Schuetz AN, et al. Multicenter evaluation of colistin broth disk elution and colistin agar test: a report from the Clinical and Laboratory Standards Institute. Journal of Clinical Microbiology. 2019;57:10-128.
14. India National Centre for Disease Control. Standard operating procedures for Colistin Agar Test for Enterobacteriaceae and Pseudomonas aeruginosa. 2020. Available from: https://ncdc.mohfw.gov.in/wp-content/uploads/2024/03/58495493521681880873.pdf
15. Jayol A, Nordmann P, Brink A et al. Heteroresistance to colistin in Klebsiella pneumoniae associated with alterations in the PhoPQ regulatory system. Antimicrob Agents Chemother 2015;59:2780-4.
16. Jeannot K, Bolard A, Plesiat P. Resistance to polymyxins in Gram-negative organisms. J Antimicrob Agents. 2017;49:526-35.
17. Karvanen M, Malmberg C, LagerbäckP, et al. Colistin is extensively lost during standard in vitro experimental conditions. Antimicrob Agents Chemother 2017;61:e00857-17.
18. Landman D, Salamera J, Quale J. Irreproducible and uninterpretable polymyxin B MICs for Enterobacter cloacae and Enterobacter aerogenes. J Clin Microbiol. 2013;51:4106-11.
19. Larrouy-Maumus G, Clements A, Filloux A, et al. Direct detection of lipid A on intact Gram-negative bacteria by MALDI-TOF mass spectrometry. J Microbiol Methods. 2016;120:68-71.
20. Meletis G, Tzampaz E, Sianou E et al. Colistin heteroresistance in carbapenemase-producing Klebsiella pneumoniae. J Antimicrob Chemother 2011;66:946-7.
21. Napier BA, Band V, Burd EM, Weiss DS. Colistin heteroresistance in Enterobacter cloacae is associated with cross-resistance to the host antimicrobial lysozyme. Antimicrobial Agents and Chemotherapy. 2014;58:5594-7.
22. Poirel L, Jayol A, Nordmann P. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. Clin Microbiol Rev. 2017; 30:557-96.
23. Schurek KN, Sampaio JL, Kiffer Cret al. Involvement of pmrAB and phoPQ in polymyxin B adaptation and inducible resistance in non-cystic fibrosis clinical isolates of Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2009;53:4345-51.
24. Simner PJ, Bergman Y, Trejo M, et al. Two-site evaluation of the colistin broth disk elution test to determine colistin in vitro activity against Gram-negative bacilli. J Clin Microbiol. 2019;57:e01163-18.
25. Stojanoski V, Sankaran B, Prasad BV, et al. Structure of the catalytic domain of the colistin resistance enzyme MCR-1. BMC Biology. 2016;14:81.
26. Uwizeyimana JD, Kim D, Lee H, et al. Determination of colistin resistance by simple disk diffusion test using modified Mueller-Hinton Agar. Ann Lab Med. 2020;40:306-11-
27. Van Belkum A, Burnham CA, Rossen JW, et al. Innovative and rapid antimicrobial susceptibility testing systems. Nature Reviews Microbiology. 2020;18:299-311.