

Frozen storage stability of pre-diluted colistin microdilution plates for routine susceptibility testing

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KEYWORDS

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Antimicrobial susceptibility testing;
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Minimum inhibitory concentration (MIC).

ABSTRACT

Broth microdilution (BMD) is the reference method recommended by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for determining colistin susceptibility testing; however, its routine implementation in clinical laboratories remains challenging due to factors such as storage conditions and technical variability. To provide a more practical and standardized approach, this study evaluated the stability of pre-diluted colistin stored at -20°C in a 96-well microdilution plate format. Serial twofold dilutions of colistin in Mueller-Hinton broth were prepared, frozen, and tested at 15-day intervals over 90 days using *Klebsiella pneumoniae* (colistin-resistant clinical isolate), *Acinetobacter baumannii* (colistin-intermediate isolate), and *Pseudomonas aeruginosa* ATCC27853 (quality control strain). Minimum inhibitory concentrations (MICs) were determined according to CLSI guidelines to monitor changes in antimicrobial activity during storage. The MIC values of all strains remained consistent throughout the 90-day period, indicating that colistin retained its biological activity under frozen storage. These findings demonstrate that colistin microdilution plates stored at -20 °C remain stable and reliable for at least three months, offering laboratories a convenient and reproducible method for preparing ready-to-use colistin BMD plates to support standardized susceptibility testing.

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Introduction

Colistin, also known as polymyxin E, is a member of the polymyxin class of antibiotics currently used in human medicine. It was first discovered in the 1940s and exhibits potent activity against gram-negative bacterial infections, particularly *Enterobacteriales*⁽¹⁾. Colistin is polycationic molecule containing both hydrophilic and lipophilic regions. These enable it to interact with the outer membrane of gram-negative bacteria by competitively displacing divalent cations, especially magnesium and calcium, from the membrane lipids. This disruption leads to increased membrane permeability, resulting in cell lysis and bacterial death. In addition, colistin can bind to and neutralize bacterial lipopolysaccharides, conferring anti-endotoxin properties⁽²⁾. Despite its effectiveness, the use of colistin declined for several decades due to reports of significant nephrotoxicity and neurotoxicity⁽²⁻⁴⁾ and the availability of safer and more potent antibiotics. However, the global emergence of multidrug-resistant (MDR) gram-negative bacteria such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*, has renewed clinical interest in colistin as a last-resort therapeutic agent⁽⁵⁻⁸⁾.

Accurate antimicrobial susceptibility testing (AST) is essential to ensure appropriate antibiotic therapy and to monitor the emergence of resistance⁽⁹⁾. Several AST methods are routinely used in clinical microbiology laboratories, including automated systems, disk diffusion, and gradient diffusion (E-test). However, both the Clinical and Laboratory Standards Institute (CLSI)⁽¹⁰⁾ and the European Committee on Antimicrobial Susceptibility Testing (EUCAST)⁽¹¹⁾ recommend broth microdilution (BMD) as the only reliable method for determining colistin susceptibility, as colistin's large cationic structure prevents accurate diffusion in agar-based assays⁽¹²⁾. Although BMD is the reference method, its use in routine clinical setting is limited by complexity of the procedure, which involves multiple

preparation steps. Factors such as inoculum preparation, culture medium, antibiotic concentration, and laboratory staff expertise can all influence the resulting minimum inhibitory concentration (MIC) values^(13,14). To make BMD more practical for routine use, laboratory could prepare pre-diluted colistin microdilution plates in advance and store them frozen. Therefore, determining the stability of colistin stored at -20 °C is critical to ensure the accuracy and reproducibility of MIC testing over time.

To our knowledge, while the stability of several reconstituted antibiotics under various storage conditions has been investigated using BMD method, similar data for colistin are lacking^(13,15,16). The present study aimed to evaluate the stability of colistin stored at -20 °C in 96-well microdilution plate format using the BMD method.

Materials and methods

Bacterial strains

Three bacterial strains were used in this study: *Pseudomonas aeruginosa* ATCC 27835 (quality control strain) and two clinical isolates, *Acinetobacter baumannii* and *Klebsiella pneumoniae*, with known colistin MIC values, obtained from the Microbiology Laboratory, King Chulalongkorn Memorial Hospital. All strains were stored at -80 °C in the Department of Microbiology, Faculty of Medicine, Chulalongkorn University.

Preparation of Colistin and bacterial suspensions

Colistin sulfate salt (Glentham Life Sciences, United Kingdom) was prepared as a stock solution at a concentration of 1,024 µg/mL in sterile distilled water. Bacterial colonies from overnight growth on sheep blood agar (Biomedica, Thailand) were suspended in 0.85% (w/v) sodium chloride solution and adjusted to a 0.5 McFarland standard (OD₆₀₀ of 0.08 - 0.10), measuring using a Bio-Rad SmartSpec™ Plus spectrophotometer; Bio-Rad Laboratories, Hercules, CA, USA), corresponding to approximate 1.5 x 10⁸ CFU/mL. The 0.5 McFarland suspension was then diluted 1:100

in cation-adjusted Mueller-Hinton broth (CAMHB; BD DIFCO™, Le Pont de Claix, France) to obtain a working inoculum of approximately 1×10^6 CFU/mL.

Evaluation of the stability of freezing colistin by using time-varying BMD

The broth microdilution (BMD) assay was performed according to CLSI guidelines⁽¹⁷⁾. Briefly, the 1024 µg/mL colistin stock solution was first diluted 1:1 in CAMHB to obtain a 512 µg/mL working solution. Serial twofold dilutions were then prepared in untreated flat-bottom 96-well microdilution plates (Wuxi NEST Biotechnology, China) by dispensing 50 µL of colistin solution in well containing 50 µL of CAMHB, resulting in colistin concentrations ranging from 0.25 to 256 µg/mL.

Following preparation of the antibiotic dilutions, 50 µL of bacterial working inoculum was added to each well, yielding final colistin concentrations ranging from 0.125 to 128 µg/mL and a final inoculum of approximately 5×10^5 CFU/mL, in accordance with CLSI recommendations.

Preparation of pre-diluted BMD trays required approximately 20-30 minutes per 96-well plate, including drug dilution and dispensing. The prepared microdilution plates were sealed, labeled, and stored at -20 °C until use. At 15-day intervals over a 90-day period, the stored plates were thawed once at room temperature (22 - 25 °C) for approximately 20 minutes and used immediately. Plates were not subjected to repeated freeze-thaw cycles, and stability was evaluated exclusively under these defined storage and handling conditions.

Each assay included a growth control well (bacteria without antibiotic; GC) and a sterility control well (broth only; SC) Figure 1. After inoculation, plates were incubated at 35 ± 2 °C for 18 - 24 hours. The MIC was defined as the lowest concentration of colistin that completely inhibited visible bacterial growth. A change in MIC by greater than ± 1 twofold dilution from the initial value was interpreted as a loss of biological activity. All experiments were performed in duplicate (technical replicates) and repeated independently three times (biological replicates).

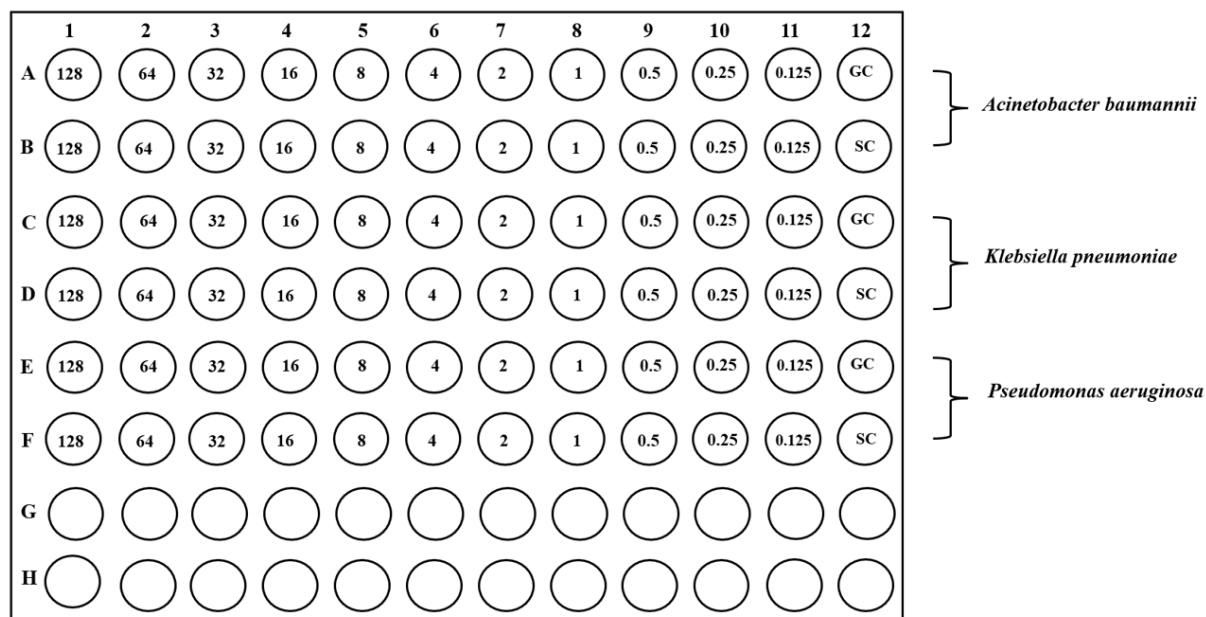


Figure 1 Layout of the 96-well broth microdilution (BMD) plate used for evaluation of colistin stability during frozen storage

The schematic illustrates the standardized plate configuration used throughout the study. Numbers indicate the final colistin concentrations ($\mu\text{g/mL}$) after addition of the bacterial inoculum, prepared as serial two-fold dilutions in cation-adjusted Mueller-Hinton broth (CAMHB), ranging from 0.125 to 128 $\mu\text{g/mL}$. Each bacterial strain was tested in two adjacent rows, with growth control wells (inoculated broth without colistin; GC) and sterility control wells (broth only; SC) included on each plate. Plates were sealed with adhesive film, stored at -20°C , and thawed at predefined time points for MIC determination.

Quality control

Quality control of inoculum density was performed in accordance with CLSI M07⁽¹⁷⁾. Briefly, 10 μL of the 0.5 McFarland suspension was transferred into 10 mL of sterile normal saline, yielding an estimated concentration of approximately 1×10^5 CFU/mL. Subsequently, 100 μL of this suspension was transferred into 10 mL of sterile normal saline, and 100 μL of the final dilution was spread on Mueller-Hinton agar (MHA; BD DIFCO™, Le Pont de Claix, France). Plates were incubated at $35 \pm 2^\circ\text{C}$ for 18 - 24 hours and colonies were enumerated. An acceptable colony count was approximately 150 colonies per plate. All assays were performed in triplicate.

Ethics statement

This study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University (IRB No. 0737/67, COE No. 063/2024).

Results

The stability of frozen, pre-diluted colistin microdilution plates was evaluated over a 90-day period using the broth microdilution (BMD) method described previously. Two clinical isolates with known colistin MIC values, *Klebsiella pneumoniae* (colistin-resistant, MIC = 64 $\mu\text{g/mL}$) and *Acinetobacter baumannii* (colistin-intermediate, MIC = 1 $\mu\text{g/mL}$), were tested along with *Pseudomonas aeruginosa* ATCC27853 as the quality control strain. Experiments were performed in technical duplicate wells and independently repeated three times on separate occasions (biological replicates). Individual MIC values from all biological replicates are shown in table 1.

Across all time points, MIC values for all strains remained within ± 1 twofold dilution of those obtained from freshly prepared microdilution plates (Table 1). For *A. baumannii*, MICs ranged from 1 to 2 $\mu\text{g/mL}$; for *K. pneumoniae*, from 32 to 64 $\mu\text{g/mL}$; and for *P. aeruginosa* ATCC27853, from 0.5 to 1 $\mu\text{g/mL}$. All values remained within one doubling dilution of the initial MIC, indicating no significant loss of colistin activity during 90 days of frozen storage at -20°C .

Table 1 Stability of pre-diluted colistin stored at -20 °C in 96-well microdilution plates determined by MIC values of test strains over 90 days

Days	MIC (µg/mL) ^a								
	<i>A. baumannii</i> (Clinical isolate)			<i>K. pneumoniae</i> (Clinical isolate)			<i>P. aeruginosa</i> ATCC27853 (0.25 - 2 µg/mL) ^b		
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
0	1/1	1/1	1/1	64/64	64/64	64/64	0.5/0.5	0.5/0.5	0.5/0.5
15	1/1	1/1	2/2	32/32	32/32	64/64	1/1	1/1	1/1
30	1/1	2/2	2/2	32/32	32/32	64/64	1/1	0.5/0.5	1/1
45	2/2	1/1	2/2	64/64	32/32	64/64	1/1	1/1	1/1
60	1/1	1/1	1/1	64/64	64/64	64/64	1/1	1/1	0.5/1
75	2/2	1/1	1/1	64/64	64/64	64/64	1/1	1/1	1/1
90	1/1	1/1	2/2	64/64	64/64	64/64	1/1	1/1	1/1

Note: ^a MIC values represent results from three independent biological replicate (Rep1 - Rep3). Value are expressed as X/X, indicating results from technical duplicate wells within the same experiment.

^b Quality Control range for *P. aeruginosa* ATCC27853 according to CLSI M100 guidelines, 2025⁽¹⁰⁾.

Discussion

Colistin is increasingly used to treat infections caused by multidrug-resistant (MDR) gram-negative bacteria, and accurate antimicrobial susceptibility testing (AST) is therefore critically important. The broth microdilution (BMD) method remains the reference procedure recommended by CLSI⁽¹⁰⁾ and EUCAST⁽¹¹⁾ for determining colistin MICs. However, routine implementation of BMD in clinical laboratories can be challenging, as the method is labor-intensive, technically demanding, and susceptible to variations introduced by media preparation, antibiotic dilution accuracy, and staff expertise. Developing a practical approach that allows laboratories to prepare and store pre-diluted colistin microdilution plates in advance would greatly enhance testing efficiency and standardization.

In this study, we demonstrated that pre-diluted colistin stored at -20 °C in 96-well microdilution plates retained its antimicrobial activity for at least 90 days, with MIC values remaining within one twofold dilution of those obtained from freshly prepared plates. Furthermore, this was confirmed by repeating the BMDs using freshly

prepared new drug lots tested on the same day as the stored plates. Minor fluctuations in MIC values were observed among the tested strains during frozen storage; however, these variations did not exceed one twofold dilution, which is within the acceptable range of variability for the BMD method⁽¹⁷⁾. Importantly, interpretive categories remained unchanged throughout the 90-day period, *A. baumannii* remained colistin-intermediate (≤ 2 µg/mL), *K. pneumoniae* remained colistin-resistant (≥ 4 µg/mL), and *P. aeruginosa* ATCC 27853 consistently fell within the established quality-control (QC) range (0.25 - 2 µg/mL). These results indicate that the antimicrobial activity of colistin was maintained during frozen storage, confirming the stability of colistin under these conditions and supporting the feasibility of preparing ready-to-use BMD trays for routine testing.

While our study validated stability across intermediate and resistant MIC ranges, isolates with MICs at or near the clinical breakpoint (2 - 4 µg/mL), such as *mcr-1*-carrying *Escherichia coli*, were not included. Because these borderline isolates are more susceptible to minor MIC shifts,

further investigation is warranted to fully confirm the robustness of pre-diluted plates for detecting low-level resistance. Future studies specifically designed to assess breakpoint robustness and limits of detection for emerging resistance mechanisms will be necessary.

Our findings are consistent with previous investigations of antibiotic stability under frozen conditions. Healan et al. reported that reconstituted colistin methanesulfonate remained stable for up to 24 hours at temperatures ranging from 21 °C to -70 °C, whereas Li et al. demonstrated that colistin was stable in aqueous solution at 4 °C and 37 °C for up to 60 days and 120 hours, respectively^(18,19). Rogacka et al. found that colistin-containing agar plates stored at 4 °C for seven days showed no change in MIC, and earlier studies reported-remarkable thermal stability of colistin at 121 °C⁽²⁰⁻²²⁾. Similar results have been documented for other antibiotics classes: Leite et al. showed that six β -lactams (meropenem, ertapenem, imipenem, ceftriaxone, ceftazidime, and piperacillin-tazobactam) could be frozen as a stock concentration for up to six months at -80 °C without a significant loss of activity⁽¹³⁾; Nickolai et al. demonstrated that 11 β -lactams (ampicillin, ticarcillin, mezlocillin, piperacillin, azlocillin, cefazolin, cefotaxime, moxalactam, cefoperazone, ceftriaxone, and imipenem) remained stable for 52 weeks at -70 °C⁽²³⁾; and Okerman et al. reported that tetracyclines and quinolones could be stored for six months at -20 °C⁽²⁴⁾. Collectively, these findings support that freezing effectively preserves antibiotic potency.

The observed stability of colistin in this study may be attributed to its chemical structure. Colistin sulfate is a cationic polypeptide that undergoes hydrolysis in aqueous solutions, a process known to be temperature-dependent⁽¹⁾. Lower storage temperatures slow hydrolytic degradation, thereby preserving structural integrity and antimicrobial activity of the compound⁽²⁵⁾. Storage at -20 °C therefore likely minimizes hydrolytic breakdown, ensuring stability for extended periods compared with storage at higher temperatures.

Most previous stability studies assessed concentrated antibiotic stock solutions that were later diluted for testing, whereas the present work specifically examined colistin that had already been diluted and distributed into microdilution trays. This approach more closely reflects real-world laboratory practice and demonstrates that pre-diluted trays remain reliable for at least three months. Frozen BMD plates can therefore be prepared in bulk, stored, and used as needed, requiring only the addition of bacterial inoculum before incubation. This strategy reduces preparation time, minimizes handling errors, and enhances inter-laboratory standardization.

Overall, this study provides new experimental evidence that colistin retains its antimicrobial activity when stored at -20 °C in a pre-diluted 96-well microdilution plate format for at least 90 days. The ability to prepare and store frozen BMD trays offers a simple, standardized, and cost-effective solution for clinical laboratories, facilitating accurate and reproducible colistin susceptibility testing in accordance with CLSI and EUCAST guidelines.

Although the number of bacterial strains evaluated was limited and the study was not designed as a full test-performance verification, the findings support the feasibility of this approach. Future studies incorporating larger numbers of clinical isolates representing intermediate, and resistant profiles, as well as isolates with MICs near the clinical breakpoint (2 - 4 $\mu\text{g/mL}$), will be necessary to further validate its applicability in routine clinical laboratories.

Conclusion

This study demonstrated that diluted colistin can be stored as a working solution at -20 °C for at least three months without loss of antimicrobial activity. The proposed approach provides a practical and user-friendly procedure for preparing pre-diluted colistin trays in advance, saving time and reducing dilution-related handling errors by laboratory personnel, thereby supporting

consistent and reproducible MIC determinations. However, verification of the biological activity of stored plates after three months of storage is recommended to ensure continued reliability.

Take home messages

This study demonstrates that pre-diluted colistin stored at -20 °C in 96-well microdilution plates remains stable and retains full antimicrobial activity for at least 90 days. These findings provide practical evidence that frozen, ready-to-use BMD plates can be reliably used for colistin susceptibility testing, reducing preparation time and variability.

Conflicts of interest

The authors declare no conflict of interest.

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Author contributions

Aschana Tirapattanuna: Data curation, Formal analysis, Investigation, Validation, Visualization, Writing - Original draft.

Pintip Suchartlikitwong: Writing - Review & Editing.

Sunisa Chirakul: Conceptualization, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Formal analysis, Writing - Review & Editing.

Data availability

Data available on request from the authors.

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