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Consultation period 11 May - 8 June 2026



CLINICAL AND
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EUCAST

European Committee
on Antimicrobial
Susceptibility Testing

Guidance for Antimicrobial Agent Developers Considering a Modification of The Standard Reference Method for Susceptibility Testing.

The final document will be published as EUCAST SOP 14.0.*

* The content in this document is identical to the content in “CLSI. *Guidance for Antimicrobial Agent Developers Considering a Modification of The Standard Reference Method for Susceptibility Testing*. 1st ed. CLSI supplement M23S4”.

Foreword

The development of new antimicrobial agents, especially those with novel mechanisms of action, is vital to combat antimicrobial resistance. A reliable antimicrobial susceptibility testing (AST) method is essential for the correct clinical use of agents and should be established early and thoughtfully to ensure timely patient access to these agents. EUCAST SOP 14.0/CLSI M23S4 offers guidance to developers of new compounds and aims to avoid unnecessary delays in new antimicrobial agent development and clinical adoption. It should be noted that any modifications can be costly and significantly delay new antimicrobial agent development and subsequent clinical. {3}

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1. Introduction

1.1 Scope

This document specifies guidance for assessing whether modification to the accepted reference method is necessary for determining the minimal inhibitory concentration (MIC) of a new antimicrobial agent for non-fastidious bacteria. This supplement is not intended to provide absolute guidance because requirements will vary from compound to compound.

The intended users of this document are developers of new antimicrobial agents at an early stage. This document is not intended for use by clinical microbiology laboratories for routine AST.

1.2 Background

Establishing a reference AST method for a new antimicrobial agent is a crucial step in early development. This enables reproducible AST to predict antimicrobial agent activity for different pathogens. Furthermore, it is a vital component used in pharmacokinetic and pharmacodynamic (PK/PD) analyses for strategies of dosing and mode of administration.

In the later stages of antimicrobial agent development and post regulatory approval, reference AST methods are utilized by diagnostic test manufacturers to establish and validate disk diffusion and other commercial tests to support proper clinical utilization of the new antimicrobial agent. Additionally, many surveillance programs and reference laboratories use the reference AST method to monitor any changes in susceptibility of the antimicrobial agent over time.

This document provides guidance for establishing data that are recommended for evaluating AST by broth microdilution (BMD) with cation-adjusted Mueller Hinton broth (CAMHB) and potential modifications of this method for new antimicrobial agents. These data are used to determine if a modified AST method is needed and if the modified AST method demonstrates the desired performance for further development.

1.2.1 Abbreviations and Acronyms

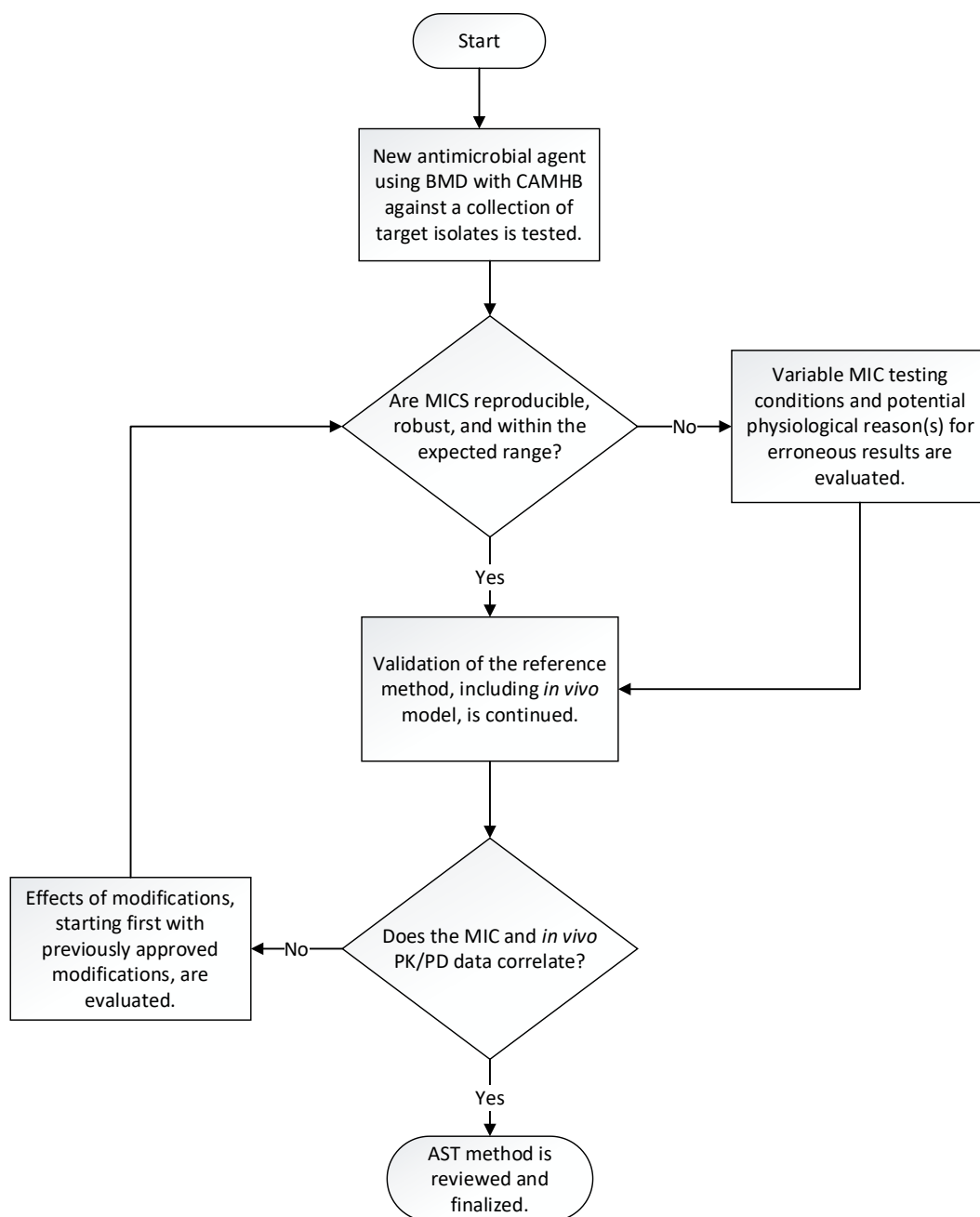
AST	antimicrobial susceptibility testing
BMD	broth microdilution
CAMHB	cation-adjusted Mueller Hinton broth
CFU	colony forming units
ECOFF	epidemiological cut-off value
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ID-CAMHB	iron-depleted cation-adjusted Mueller Hinton broth
ISO	International Standards Organization
MIC	minimal inhibitory concentration
PD	pharmacodynamic
PK	pharmacokinetic
QC	quality control
SDO	standards development organization

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2. Process Flowchart for Evaluating AST Methods

Figure 1 is a flowchart detailing the necessary decision points when evaluating a reference minimal inhibitory concentration (MIC) method for broth microdilution (BMD) in cation-adjusted Mueller-Hinton broth (CAMHB).

Figure 1. Process Flowchart for Evaluating AST Methods



Abbreviations: AST, antimicrobial susceptibility testing; BMD, broth microdilution; CAMHB, cation-adjusted Mueller Hinton broth; MIC, minimal inhibitory concentration; PK/PD, pharmacokinetic/pharmacodynamic.

^a 4 basic symbols are used in this process flow chart: oval (signifies the beginning or end of a process), arrow (connects process activities), box (designates process activities), diamond (includes a question with alternative "Yes" and "No" responses).

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3. Guidance for Evaluation of AST Methods

Both US and European regulatory agencies recommend that the methods used for MIC determination are described in detail and justified. ^{6}^{7} If a modified method is needed, it is advisable that antimicrobial agent sponsors justify the change by collecting comparative data with the reference antimicrobial susceptibility testing (AST) method and alternative methods. Analyses should include MICs determined using the reference AST method and with the proposed modification for strains tested in animal infection models. Additionally, the pathogens tested should include a spectrum of 'resistance' phenotypes to ascertain whether the exposure-response relationship is affected by a specific 'resistant' subgroup or is generalized across all isolates with different 'resistance' mechanisms. Collection of comprehensive, comparative data is recommended to support adoption of a modified method by standards development organizations (SDOs).

3.1 Lack of Reproducibility of the AST Method

One reason for considering a modified AST method is due to the consistent lack of reproducibility in a robust MIC data evaluation. Irreproducible MICs may be due to several reasons, for example trailing endpoints, skipped wells, medium interference, instability of the antimicrobial agent in the test medium (eg, CAMHB) and inoculum effects.

The reproducibility of an MIC test refers to the extent to which consistent MICs are obtained when the test is repeated. ^{8} Repeat testing of an antimicrobial agent against a single quality control (QC) strain with the reference broth microdilution method, without varying the medium source or other components, should yield MICs within 2, or a maximum 3, doubling dilutions, with MIC values being within 1 doubling dilution of the median or modal MIC (see examples in Table 2).

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Table 2. Modified Reference AST Methods Recognized by a Standards Development Organization

Antimicrobial Agent	Organism Group	Method	Medium	SDO	Justification
Cefiderocol	Non-fastidious Gram-negative organisms	BMD	Iron-depleted CAMHB (ID-CAMHB)	CLSI M07 ^{1} CLSI M100 ^{9} EUCAST ^{10}	Iron chelation essential for siderophore uptake mechanism
Daptomycin	Gram-positive cocci	BMD	CAMHB + 50 µg/mL Ca ²⁺	CLSI M07 ^{1} CLSI M100 ^{9} EUCAST ^{11}	Calcium required for activity
Tigecycline	Gram-positive organisms and Gram-negative organisms	BMD	CAMHB, fresh medium preparation	CLSI M07 ^{1} CLSI M100 ^{9} EUCAST ^{11}	Antimicrobial agent instability in aged medium
Oritavancin Televancin Dalbavancin	Gram-positive cocci	BMD	CAMH broth + polysorbate-80 (0.002 %)	CLSI M07 ^{1} CLSI M100 ^{9} EUCAST ^{11}	Polysorbate-80 required to ensure accurate MICs and prevent binding to plastic

Abbreviations: BMD, broth microdilution; CAMHB, cation-adjusted Mueller Hinton broth; EUCAST, European Committee on Antimicrobial Susceptibility Testing; MIC, minimal inhibitory concentration; SDO, standards development organization.

Reproducibility of an AST method for antimicrobial agent in development should be investigated by repeat testing of QC strains. This testing may include testing of the QC strain with a comparator agent with published QC ranges to evaluate if the strain is behaving as predicted. Poorly reproducible MIC results with QC strains may indicate an AST issue even before a larger panel of organisms are tested. However, even if reproducible QC results are obtained using BMD with CAMHB, a large panel of clinical isolates should still be evaluated as soon as is reasonably possible because QC strain MIC reproducibility may not reflect the variability of MICs against clinical isolates. This should include clinical isolates of species relevant to the agent in a robust evaluation. Ideally, isolates representing both wild-type and non-wild type MICs of relevant species should be selected for reproducibility analysis. These isolates can be chosen by evaluating epidemiological cut-off values (ECOFFs). ^{12} This is important as it investigates the reproducibility over a large part of the MIC scale. If the magnitude of variation exceeds that described above, or if MICs distribute over 3 or more doubling dilutions without having a clear median or mode, the reproducibility should be further investigated.

In later stages of antimicrobial agent development, when an MIC method has been established, QC studies are performed according to CLSI M23 ^{13} or EUCAST ^{14} guidelines to establish acceptable limits for specific strain-antimicrobial agent combinations. CLSI and EUCAST MIC ranges comprise 3, and sometimes 4, doubling dilutions and are established to include reasonable variation between laboratories and media manufacturers. If MICs for the antimicrobial agent are not reproducible when tested with a single CAMHB manufacturer, or in

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a single laboratory, problems will certainly arise when establishing QC ranges, clinical breakpoints and for laboratories performing the AST.

3.2 Possible Reasons for Irreproducible MICs and Suggestions on How to Investigate

There are several reasons for poor reproducibility of MIC determination with reference BMD, some of which may remain unknown. Regardless, it is always advisable to perform tests to identify the reason(s) for the irregular MICs.

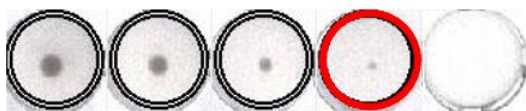
3.2.1 Trailing Endpoints

Some antimicrobial agent-organism combinations may produce trailing endpoints with a gradual fading of growth, making MIC determination difficult. CLSI M07 ^{1} and EUCAST ^{15} have specific recommendations for how to read MIC endpoints when trailing occurs. Examples are ignoring tiny buttons of growth for certain, normally bacteriostatic, agents, (Figure 2A) and reading the MIC at the lowest concentration that inhibits $\geq 80\%$ of growth as compared to the growth control for trimethoprim and the sulphonamides (Figure 2B).

If trailing endpoints occur for the antimicrobial agent in development, it should be investigated if alternative reading instructions, and primarily those already recommended by CLSI and EUCAST, produce more reproducible MICs rather than modifying the test medium.

Figure 2. Trailing Endpoints (Reprinted with permission from EUCAST ^{15})

A)



B)



A) Ignoring tiny buttons of growth for linezolid. B) Reading trimethoprim-sulfamethoxazole MICs at $\geq 80\%$ inhibition as compared to the growth control. MIC result is indicated by a red circle.

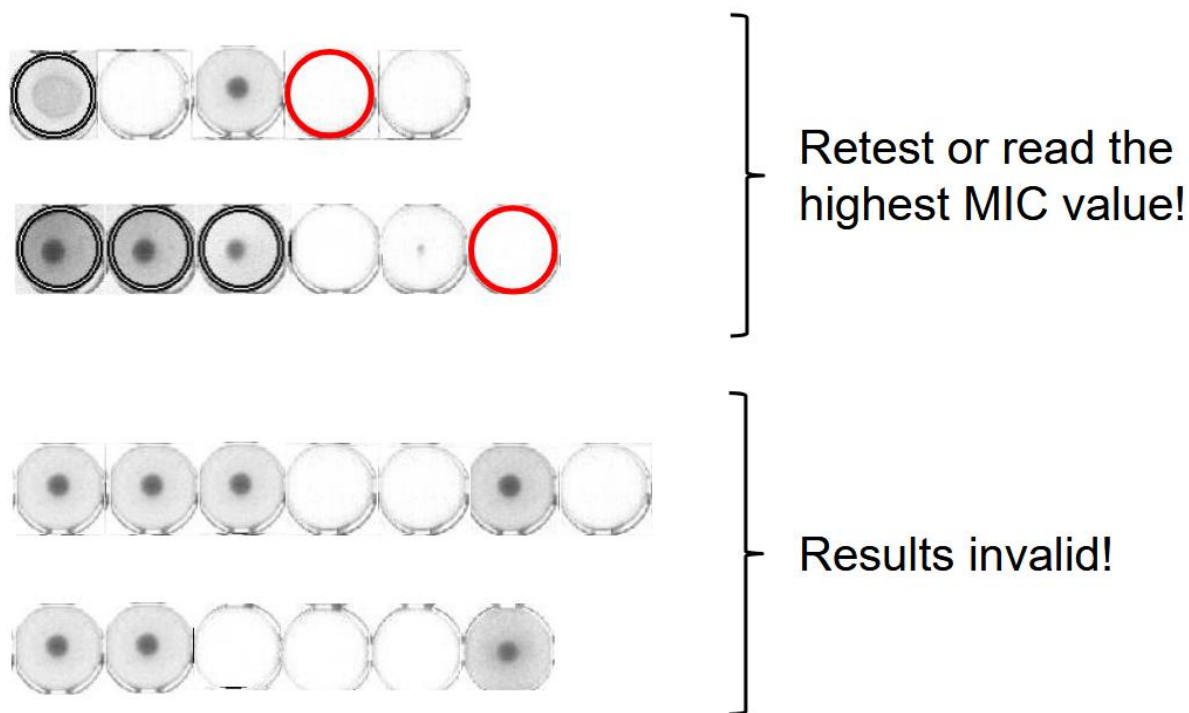
3.2.2 Skipped Wells

It is not unusual that a 'skipped well', a well of no bacterial growth followed by one or several wells with growth, may occur when testing a dilution series of any antimicrobial agent (old or new). There are several possible explanations including incorrect inoculation, contamination, heterogenous resistance, and plate preparation errors. Examples are shown in Figure 3. ^{15} However, if skipped wells repeatedly occur, resulting in many MIC test results being invalid

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when testing a new antimicrobial agent, there may be a need to modify the testing method to overcome this problem.

Figure 3. Skipped Wells (Reprinted with permission from EUCAST ^{15})



Abbreviation: MIC, minimal inhibitory concentration.

3.2.3 Medium Interference

Some antimicrobial agents are more affected by the composition of the test medium than others. BMD shall be performed using CAMHB and when needed, modified as described in Table 2 to achieve sufficient growth for some species or to accurately measure antimicrobial activity. ^{1} ^{2} The CAMHB used should meet the specifications in international standards. ^{16} If irreproducible MICs are observed when performing BMD with CAMHB from 1 manufacturer, CAMHB from additional manufacturers should be tested to investigate if this is related to the CAMHB brand used. If preliminary data suggests that a modification of the reference method is needed, then modifications already approved by CLSI and EUCAST should be evaluated first (see Table 3).

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Table 3. Example of reproducible MIC distributions from 10 repeats of broth microdilution for three antimicrobial agents tested against one QC strain in a single laboratory using cation-adjusted Mueller Hinton broth from 1 manufacturer

MIC (µg/mL)	Agent A	Agent B	Agent C
0.008			
0.016	2		
0.03	6		
0.06	2		
0.125		1	
0.25		8	
0.5		1	
1			
2			4
4			6
8			
Median	0.03	0.25	4
Mode	0.03	0.25	4

Abbreviations: MIC, minimal inhibitory concentration.

3.2.4 Instability of the Antimicrobial Agent in the Test Medium

Instability of the antimicrobial agent in the test medium is another possible reason for irreproducible MICs. Tests to investigate this should include testing different solvents, diluents, or different salts of the active antimicrobial agent. Investigations into possible supplementation or modification of CAMHB should be secondary to these tests.

3.2.5 Inoculum Effect

The target final inoculum in BMD is 5×10^5 CFU/mL with an acceptable range of 2×10^5 to 8×10^5 CFU/mL. ^{{1}{2}} Modifying the inoculum is not suggested as a modification of the AST method but could be an important part of investigating irregular MICs. When assessing the inoculum effect, it is advisable to start by determining if small differences within the specified inoculum range (2×10^5 to 8×10^5 CFU/mL) influence the MICs.

3.3 Unexpected Results from the AST Method

Early screening of new antimicrobial agents is often limited. For example, testing may involve a truncated antimicrobial concentration range or even a single test concentration to quickly assess the activity of a large panel of antimicrobial agents against a small number of target organisms. Furthermore, for convenience or historical reasons, this initial screening may not use the same medium as defined in the reference AST method.

Once a potential lead antimicrobial agent, or a shortlist of candidates, is found it is important to evaluate antimicrobial agent activity against a larger panel of organisms (as noted in Subchapter 3.1) and test a broader range of dilutions (a typical range of doubling dilution

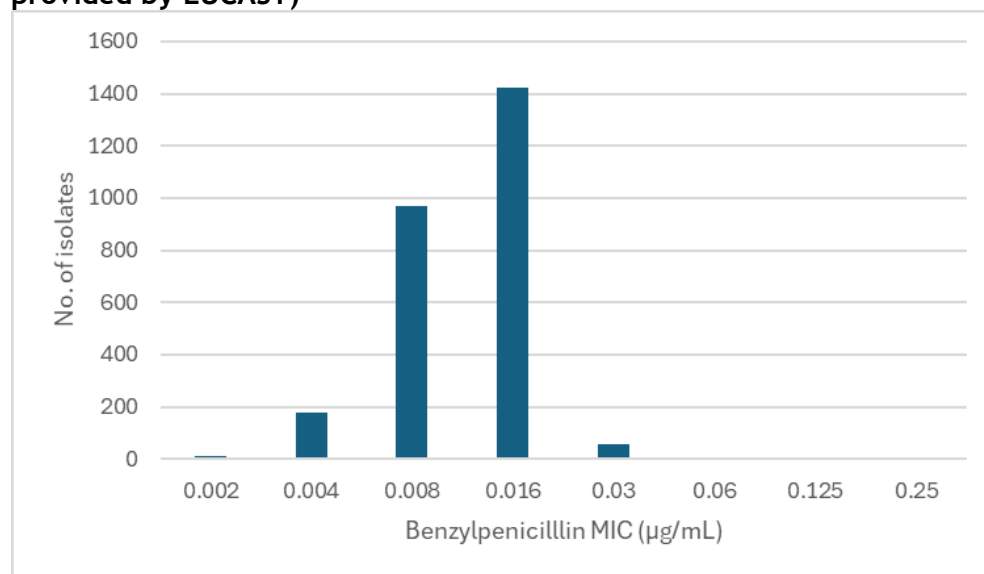
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concentrations is from 0.008 µg/mL to 128 µg/mL). This panel should include a wider selection of species than originally screened, isolates from diverse origins as well as antimicrobial agent-resistant isolates where possible. This analysis should be performed at an early stage in development using BMD with CAMHB to measure the relative activity of candidate antimicrobial agents and as an initial check of performance using this method, especially if the early screening used a different medium. Erroneous results from these studies may be a first signal that a method change could be required.

MIC studies should always include relevant QC strains, even though official ranges for the new antimicrobial agents will not have been established. It is important to evaluate the activity of a new antimicrobial agent multiple times against QC organisms to evaluate the reproducibility of the new antimicrobial agent QC using BMD with CAMHB. It is also good practice to do this for each new antimicrobial agent lot tested and to record the cumulative QC MIC results throughout antimicrobial agent development. This is discussed in Subchapter 3.1 on reproducibility.

When an antimicrobial agent is tested against many isolates from a single species most MICs should fall within a relatively narrow range in a gaussian-type distribution around a mode value. An example of this for benzylpenicillin against *Streptococcus pyogenes* is shown in Figure 4. A clear mode MIC of 0.016 µg/mL is observed, with MICs for other isolates without phenotypic resistance straddling this value (ie, wild-type isolates). Clinical isolates with MICs at higher concentrations (ie, non-wild type isolates) may also occur with established antimicrobial agents due to resistance development over time. An example of this for erythromycin against *Staphylococcus aureus* is shown in Figure 5. Despite resistance development, the core erythromycin MIC distribution still retains a gaussian MIC distribution for wild-type isolates with MICs from 0.06 µg/mL to 1 µg/mL and a dominant mode of 0.25 µg/mL.

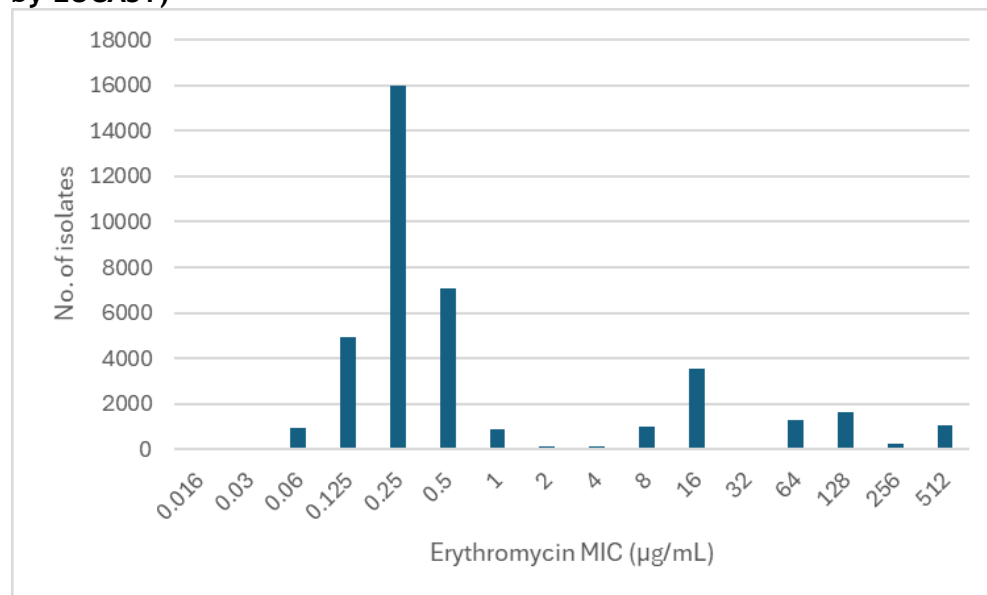
Figure 4. MIC Distribution for Benzylpenicillin Against *Streptococcus pyogenes* (Data provided by EUCAST)



Abbreviation: MIC, minimal inhibitory concentration.

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Figure 5. MIC Distribution for Erythromycin against *Staphylococcus aureus* (Data provided by EUCAST)

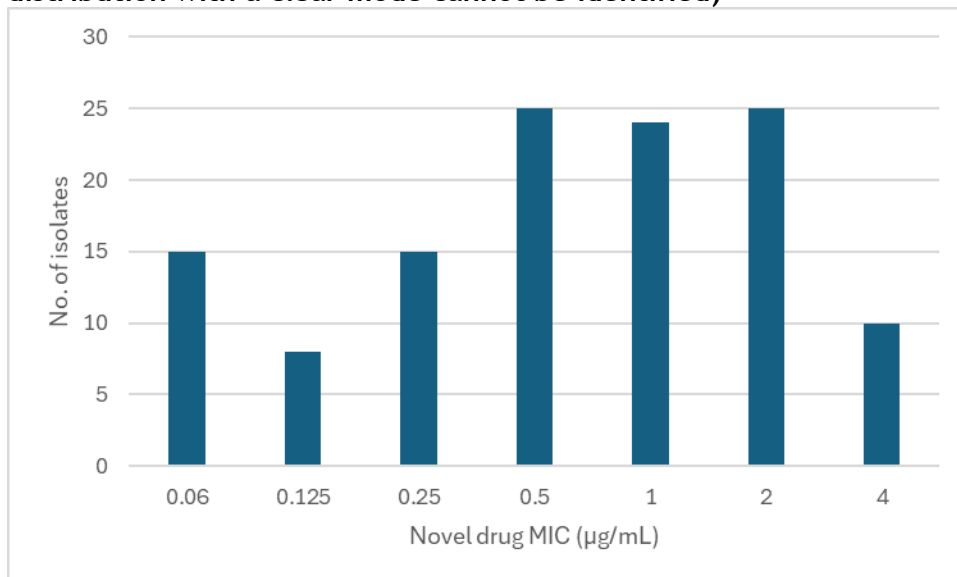


Abbreviation: MIC, minimal inhibitory concentration.

The expectation is that novel antimicrobial agents will also show a gaussian-type MIC distribution with few 'resistant' (non-wild type) isolates, if any at all. If this type of MIC distribution is not obtained using BMD with CAMHB, this may indicate that there is a problem with the antimicrobial agent (eg, poor solubility, instability) or a difficulty with the testing method itself. A hypothetical example of an unexpected MIC distribution is shown in Figure 6. A flat MIC distribution indicates that differentiation between susceptible and resistant isolates would be very difficult with such a wide variability in MIC results for presumed wild-type isolates. Further investigations are required to eliminate antimicrobial agent-associated issues (eg, improve solubility by use of a different chemical salt of the antimicrobial agent or a different solvent) as the cause of the poor AST problem before seeking an alternate method, as described in Subchapter 3.1.

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Figure 6. Hypothetical MIC distribution for a novel antimicrobial agent against a single organism species with potential method problems (eg, a gaussian-shaped wild-type distribution with a clear mode cannot be identified)



Abbreviation: MIC, minimal inhibitory concentration.

Before, or as part of proposing a modified method for MIC testing, a distribution of reference AST method MICs for relevant target species should be presented and analyzed. As seen in the examples, for each species a gaussian shape distribution with a width of 3 - 5 2-fold concentrations is considered acceptable. Any proposed modified method would be expected to resolve the MIC distribution and reproducibility problem as well as satisfy the other criteria discussed in CLSI M23S4.

3.4 *In Vivo* Pharmacodynamic Studies

The goal of establishing an AST reference method for an antimicrobial agent is to provide objective, quantitative data (eg, an MIC) to support the selection of appropriate therapy (based on susceptibility interpretive criteria). As the BMD MIC assay is the reference method recognized by CLSI, ISO, and EUCAST, it should be assessed initially. However, some antimicrobial agents may require alternate physiologically relevant testing conditions to establish robust and reproducible MICs. Pharmacodynamic (PD) studies (*in vitro* and *in vivo* infection models) are recommended as early as possible during development to identify exposure-response relationships and to establish if the microbiological measure of susceptibility (ie, MIC) provides translation to *in vivo* efficacy. In this subchapter, potential modifications to the BMD methodology related to *in vivo* antimicrobial activity are described.

A modified BMD reference method for a new antimicrobial agent may need to be considered when exposure-response relationships obtained *in vivo* are discordant with MIC values generated using the reference AST method (ie, no correlation between MIC and efficacy relative to *in vivo* exposure). When this occurs, reasons for the lack of an exposure-response relationship must be investigated. When *in vivo* efficacy exceeds that suggested by MIC values, several factors including the physiological conditions under which the MIC is determined may be responsible (eg, medium ion concentrations, loss of active antimicrobial agent due to binding

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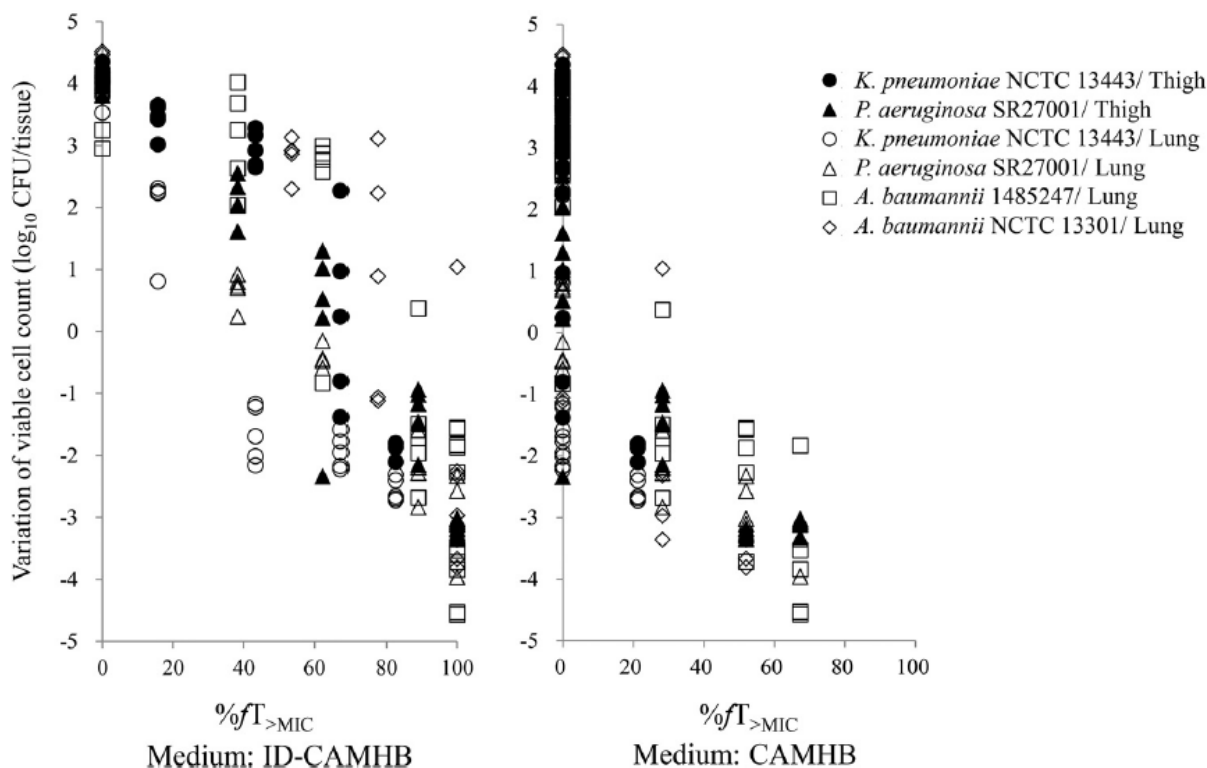
to plastic, antimicrobial agent solubility; see Subchapter 3.1). When *in vivo* activity is lower than that predicted by the MIC, various factors including formulation challenges, inter- and intra-species variation in pharmacokinetic (PK) attributes such as exposure at site of infection, *in vivo* inactivation, protein binding, and plasma or infection site-specific PK parameter estimates warrant consideration.

In studies aimed to identify the PK/PD exposure-response relationship associated with reductions in bacterial burden, PD parameters such as area under the concentration-time curve over 24 hours ($fAUC_{0-24}$), and maximum free antimicrobial agent concentration (fC_{max}) are indexed to the MIC to derive the AUC/MIC and fC_{max}/MIC . Furthermore, the percentage of time of the dosing interval in which the free antimicrobial agent concentration is above the MIC ($\%fT > MIC$) may also be applicable. PK/PD target assessment is dependent on accurate MIC determination; therefore, MIC values that do not accurately reflect the true potency of the antimicrobial agent may lead to erroneous magnitudes of the various PK/PD indices needed to achieve stasis, or 1- or 2- \log_{10} reductions in bacterial counts in animal models. In turn, errors in setting non-clinical targets may result in selecting a suboptimal clinical dose. Alternative PK/PD indices should also be explored in the absence of observed exposure-response relationships, because MICs using the reference AST method is often still effective, but increased potency (ie, extremely low MICs) creates challenges in separating standard PK/PD indices on the low range. For example, the use of AUC/MIC corrected for dosing interval, $fT > \text{threshold concentration (Ct)}$, or $fT > Ct/MIC$ are novel PK/PD indices that have established an improved exposure-response relationship over standard PK/PD indices while still employing the reference AST method with tebipenem and imipenem/meropenem.^{{17}{18}}

A comprehensive analysis performed with the novel siderophore cephalosporin cefiderocol provides a relevant example of *in vivo* PD activity exhibiting a poor fit with MICs determined using the reference BMD assay.^{19} Cefiderocol contains a catechol moiety that chelates iron, enabling bacterial uptake across the outer membrane via membrane-bound iron transporter channels.^{20} As iron is a critical component of cefiderocol antimicrobial activity, the presence of elevated concentrations of iron in CAMHB can increase the MIC obtained in the BMD assay.^{21} Thus, the modified reference method for cefiderocol utilizes iron-depleted CAMHB to obtain more reliable and reproducible MIC results under conditions that better reflect physiological iron concentrations at sites of infection in humans.^{1} Analysis of data obtained in mouse neutropenic thigh and lung infection models showed that a better fit of the PD data was obtained when indexed to the MICs of challenge pathogens obtained in iron-depleted CAMHB (ID-CAMHB) relative to MICs obtained using the reference AST method in CAMHB, see Figure 7.^{19} As shown in the analysis, bacterial killing was observed against target pathogens exhibiting high MICs determined in CAMHB (range: 32 mg/L to 256 mg/L), contrasting the MICs obtained in ID-CAMHB (range: 1 mg/L to 16 mg/L). These results highlight the need for diligence when assessing the PD activity in animal models of infection. For example, bacterial growth is to be expected under conditions where free drug levels are below the MIC (ie, zero $\%fT > MIC$), which is exactly the result observed when cefiderocol MIC values determined in ID-CAMHB were used in the PK/PD calculations (see Figure 7 left panel). In contrast, when cefiderocol MIC values determined in CAMHB were used in PK/PD calculations the data showed substantial bacterial killing even with target pathogens at zero $\%fT > MIC$ (see Figure 7 right panel). This unexpected result occurred because MICs determined in CAMHB were too high.

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Figure 7. Comparison of the %T_{>MIC} of cefiderocol between MIC values in iron-depleted cation-adjusted Mueller Hinton broth (ID-CAMHB) and cation-adjusted Mueller Hinton broth (CAMHB) in neutropenic murine thigh and lung infections ^{19}



Abbreviations: CAMHB, cation-adjusted Mueller Hinton broth; CFU, colony forming unit; ID-CAMHB, iron-depleted cation-adjusted Mueller Hinton broth.

4. Unnecessary Modification of the AST Method

Modification of the reference BMD method is a significant undertaking that can delay development, but more importantly, a modification can limit testing and therefore ultimately reduce use of the antimicrobial agent for patient care. This is because modification of the reference AST method often means that AST device manufacturers require more resources and time to develop a commercial diagnostic test. In some cases, a commercial diagnostic test may not be technically feasible. In other cases, the test may only be available as a stand-alone test, which increases the supply, labor costs, and time required for testing. Clinical microbiology laboratories cannot perform the testing needed to identify infections that are likely to respond to the new antimicrobial agent if commercial tests are delayed, unavailable, or costly. ^{3}

The following reasons do not justify the modification of an AST method:

- **Modifying the AST method without sound physiological justification.** The physiological justification is a rationale based upon the antimicrobial agent's mechanism of action and data, described above, indicating that modification of the AST method is necessary for accurate MIC determination.

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- **Lowering MIC values to demonstrate greater activity than competitor antimicrobial agents.** There may be multiple drivers or misunderstandings that can result in this activity. The following are two clarifications that can help to address concerns.
 - Irrespective of the method used, the MIC is a relative value, and the activity of an antimicrobial agent cannot be determined solely by MIC values. Instead, activity is determined by applying data derived from relevant *in vitro* and *in vivo* infection models to MIC data. ^{13}
 - Some antimicrobial agents, like peptides, have a higher molecular weight than traditional small molecule antimicrobial agents. This results in a higher MIC per molecule when the MIC is expressed as µg/mL or mg/L, both traditional expressions of MIC values. For educational purposes, this problem can be addressed by expressing the MIC in both weight per volume units and molar units per volume when questions regarding MIC values occur.
- **Modification of the AST method to support growth of both fastidious and non-fastidious bacteria.** It is preferred to use BMD with CAMHB for non-fastidious aerobic bacteria and an accepted reference method for fastidious and anaerobic bacteria. AST methods for fastidious and anaerobic bacteria can be found in CLSI M100 ^{9}, CLSI M45 ^{22}, and EUCAST Clinical Breakpoint Tables. ^{11}

5. Conclusion

Several *in vitro* AST studies and *in vivo* correlation experiments are required to determine if a modification of the reference method is required or not as discussed in detail above. It is important to recognize that each antimicrobial agent is unique and may present other challenges not specified in CLSI M23S4. A full assessment of the pros and cons of a modified method should be made because modifications of the reference method may well have a detrimental effect on the future development of susceptibility test devices. It is recommended that antimicrobial agent developers consult with SDOs early in the decision-making process.

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