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Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guideline

This document provides procedures for determining the lethal activity of antimicrobial agents.

A guideline for global application developed through the Clinical and Laboratory Standards Institute consensus process.

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Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guideline

Abstract

Established laboratory methods that can assess the bactericidal activity of an antimicrobial agent are needed, both because of the increase in the number of patients who do not have completely normal host immune defenses and because of the new classes of antimicrobial agents that have been introduced. Clinical cure depends largely upon host factors. Bactericidal tests can provide a rough prediction of bacterial eradication. It should be noted, however, that other factors (e.g., postantibiotic effect and the growth-inhibitory effects of sub-MIC concentrations of antibiotics) may also impact bacteriologic response of patients. The special susceptibility tests that assess lethal activity are not routinely applied to all microorganisms, but are applied in unusual situations; e.g., endocarditis. Uniform test procedures are thus needed to permit comparison of different datasets.

The methods for bactericidal testing are now evolving, but more work is needed with the methodological aspects and clinical correlations. The techniques described in this document are intended primarily for testing aerobic bacteria that grow after incubation in adjusted Mueller-Hinton broth or adjusted Mueller-Hinton broth supplemented with human serum or an ultrafiltrate thereof.

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Contents

Contents (Continued)

Foreword

All of the susceptibility test methods commonly performed by clinical microbiology laboratories (e.g., disk diffusion, broth dilution, and agar dilution) measure the inhibitory activity (MIC) of an antimicrobial agent.[1,2,3] In most clinical situations, this is sufficient as the role of the antibiotic is to prevent the spread of bacteria from the focus of infection by preventing microbial replication at new sites; the active participation of the host's defense mechanisms finally achieves bacterial eradication and clinical cure.[4] Antimicrobial assays can provide additional valuable information on the pharmacokinetics of the agent(s) being used and, when combined with the MICs, can allow bacterial eradication to be predicted.

On occasion, it may be necessary to achieve bactericidal activity with an antimicrobial agent. This need has been well documented for endocarditis[5] and has been suggested by some for meningitis,[6] for osteomyelitis,[7] as well as for infections in immunocompromised patients.[8] The clinical occurrence of tolerance[9] may on rare occasion necessitate bactericidal testing.

When assessment of bactericidal activity is deemed appropriate, an *in vitro* test method such as the MBC determination or the use of time-kill kinetic methodology may be useful. Bactericidal activity against the patient's isolate by the antibiotic tested allows eradication to be predicted based upon the usual dosing of this antibiotic or based upon the results of an antimicrobial assay. When clinical experience is lacking and assay methods are not readily available, the serum bactericidal test which integrates both pharmacodynamic and pharmacokinetic properties may be more useful. Depending on certain modifications to the serum bactericidal test, the test can provide a quantitative assessment of bactericidal activity relative to the MBC (the serum bactericidal titer), a dynamic assessment of rapidity of killing over time (the serum bactericidal rate), or both the magnitude of serum bactericidal activity and its duration (the area-under-the-bactericidal-titer-curve). In addition, methods using serum from persons (e.g., volunteers) receiving antibiotics (*ex vivo*) can be used to assess antimicrobial bactericidal activity across drug classes or between members of a class against a wide variety of microorganisms.

Because of the complexity involved with the serum bactericidal test (including the particular method used, the proper collection of timed serum specimens, and the interpretation of results), and the lack of clinical data clearly documenting the usefulness of this test for most infections, it is recommended that consultation with the microbiology laboratory be obtained as a prerequisite for this test. The assistance of the laboratory's director is useful in (1) determining if such a test is needed; (2) selecting NCCLS recommended methodology for testing; and (3) interpreting the results. Techniques for the conduct of the serum bactericidal test may be found in the most current edition of NCCLS document M21—*Methodology for the Serum Bactericidal Test*.

This document describes the details of bactericidal testing and, in particular, the effects of variations in methodology. This information has been obtained largely from published data. Use of these guidelines should result in uniform methodology for bactericidal testing that is sufficiently practical for use in the clinical microbiology laboratory. The methodology described in this document does not imply *per se* that bactericidal testing is clinically relevant, but instead allows such testing to be used as a tool to assess clinical relevance. The techniques described in this document are intended primarily for testing aerobic bacteria that grow well after overnight incubation in either Mueller-Hinton broth or in Mueller-Hinton broth supplemented with human serum as described in Section 2.2.1. Modifications for more fastidious microorganisms such as anaerobes will be described in detail in the future.

Standard Precautions

Because it is often impossible to know what might be infectious, all human blood specimens are to be treated as infectious and handled according to "standard precautions." Standard precautions are new guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of any pathogen and thus are more comprehensive than universal precautions which are intended to apply only to transmission of blood-borne pathogens. Standard precaution and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (Guideline for Isolation Precautions in Hospitals, Infection Control and Hospital Epidemiology, CDC, Vol 17;1:53-80.), [MMWR 1987;36(suppl 2S):2S-18S] and (MMWR 1988;37:377-382, 387-388). For specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials; and recommendations for the management of blood-borne exposure, refer to NCCLS document M29—*Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue*.

Key Words

Bactericidal activity, minimal bactericidal concentration (MBC), minimal lethal concentration, serum bactericidal concentration, time-kill determination

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1 Introduction

1.1 Rationale

An increasing number of patients with infections do not have completely normal host immune defenses. In addition, classes of antibiotics that have been considered to be bactericidal can no longer be assumed to kill every clinical isolate due to the possibility of tolerance.[9] Finally, as new classes of antimicrobial agents are introduced, there is a need for established laboratory methods that can assess the bactericidal activity of these agents. Such methods for assessing lethal activity should be considered special susceptibility tests because they are not routinely applied to all microorganisms; rather, they are applied in unusual situations. Because of their specialized nature, complexity, and potential difficulty for interpretation, tests for bactericidal activity should be done in the context of consultation with appropriate persons (such as the microbiology laboratory director) who are aware of the potential problems involved in such testing.

1.2 Methods

The killing effect of an antimicrobial agent on a microorganism can be assessed in several ways:

(1) Lethal activity may be expressed as the rate of killing by a fixed concentration of drug under controlled conditions. This rate is determined by measuring the number of viable bacteria at various time intervals. The resulting graphic depiction is known as the time-kill curve. Bacterial killing rates are, in part, dependent on the class of antibiotic and the concentration of this agent. With certain classes of antibiotics (e.g., aminoglycosides and fluoroquino-lones), the rate of killing increases with increased drug concentrations up to a point of maximum effect.[10] This is termed *concentration-dependent bactericidal activity*. In contrast, the killing rates of β-lactam agents and vancomycin are relatively slow and continue only as long as the concentrations are in excess of the MIC.[10,11,12] This rate of killing is termed *time-dependent bactericidal activity.*

(2) The minimal concentration of drug needed to kill most (≥99.9%) of the viable organisms after incubation for a fixed length of time (generally 24 hours) under a given set of conditions is the most common estimation of bactericidal activity and is known as either the minimal bactericidal concentration (MBC) or the minimal lethal concentration (MLC). It will be referred to in this document as the MBC. Unfortunately, the definition of the MBC (99.9% killing of the final inoculum) is somewhat arbitrary and separates the bacteria into two populations – a segrega-tion which might not have biological relevance.[13] The determination of the MBC, moreover, is so subject to methodologic variables that the clinical relevance of MBCs is nearly impossible to assess, particularly for certain pathogen and drug combinations (e.g., staphylococci and beta-lactam agents).[14]

(3) The serum of a patient receiving an antibiotic may be tested against the infecting microorganism. This can be done using time-kill curve methodology (i.e., serum bactericidal rate) or using dilution methodology (i.e., serum bactericidal titer). The principles of these methods as well as the influence of biological and technical factors are similar.

1.3 Problems

Evaluating *in vitro* lethal effects of an antimicrobial agent is conceptually attractive and appears, at times, to be clinically necessary. However, many biological and technical factors are known to interfere with such *in vitro* measurement of killing.

The biological factors include:

- Persisters
- Paradoxical effect
- Tolerance

- Phenotypic resistance.

The technical factors include:

- Growth phase of inoculum
- Inoculum size
- Insufficient contact
- Volume transferred
- Antibiotic carryover
- Choice of media.

These variables create uncertainty in the interpretation of bactericidal activity and are a major reason that professional consultation with the microbiology director is needed.

1.3.1 Biological Factors

1.3.1.1 Persisters

For some well-studied antibiotics, e.g., β-lactam agents, a small number (usually <0.1% of the final inoculum) of bacteria were found to survive the lethal effect of an antibiotic.[15] If these persisters are retested, they are just as susceptible as the parent strain and no greater proportion of cells persist. This phenomenon is thought to be due to the fact that some cells are dormant or replicating slowly and consequently are not killed by the antimicrobial agent.[16] The rate of antimicrobial agent-induced killing is strictly proportional to the rate of bacterial growth.[17] Thus, the slower the rate of bacterial growth, the slower the bactericidal effect of the antibiotic. As the growth of a microorganism reaches its maximum, the rate slows and so does the killing effect.

1.3.1.2 Paradoxical Effect

Another factor known as the *paradoxical effect* occurs when the proportion of surviving cells increases significantly as the concentration of the antimicrobial agent increases beyond the MBC.[18,19] This phenomenon is particularly common for cell wall-active agents. It is thought that a high concentration of penicillin inhibits protein synthesis to a degree which prevents the growth necessary for expression of the lethal effect of the drug. Penicillin also has been found to lyse RNA[20] and this might be related to the paradoxical effect.

There is also another mechanism of penicillin-induced cell death which is not related to lysis[21]; the relationship of this mechanism to the paradoxical effect is unknown. A paradoxical effect of aminoglycosides on the growth of gram-negative bacilli also has been described.[22]

The clinical relevance of the paradoxical effect is unclear. However, Eagle and his coworkers[23] infected mice with group B streptococci and demonstrated that the bacteria were killed *in vivo* more slowly by high doses of penicillin. There is at least one reported case[24] where a reduction in dosage of penicillin (peak levels decreasing from 36.7 to 11.3 µg/mL) resulted in a marked increase in bactericidal activity in the patient's serum (peak titers increasing from 1:8 to 1:256) with coincident improvement in clinical status.

1.3.1.3 Tolerance

Tolerance means that the microorganism is able to evade only the lethal action of the antimicrobial agent; there is no change in the MIC.[9,18,25-27] At least four mechanisms have been described which enable clinical isolates to survive during therapy with cell-wall-active agents. Two of these, persisters and the paradoxic effect, have already been described.

Another mechanism is phenotypic tolerance.[16] Phenotypic tolerance is a property of virtually all strains of bacteria and is defined as decreased susceptibility to antimicrobial agents which is manifested only under certain growth conditions.

The last mechanism of tolerance is that in which a microorganism possesses or acquires a unique genetic property, such as a defective autolytic system.[27]

All tolerant isolates, no matter which mechanism is responsible, exhibit unusually high MBCs relative to their MICs, and tolerance has been defined as an MBC-MIC ratio of 1:32 or greater after 24 hours of incubation. However, such a ratio cannot distinguish phenotypic tolerance from tolerance due to a unique genetic property. Instead, a time-kill kinetic study is needed to differentiate these

two mechanisms of tolerance. In a time-kill study, a phenotypically tolerant isolate demonstrates an initial high rate of killing similar to that of a nontolerant isolate, but then reaches a higher survival rate (> 0.1%), whereas tolerance due to a unique genetic property is characterized by a slow loss of viability during the first 24 hours of the entire 48-hour killing curve. Yet, both will have a high MBC and an MBC-MIC ratio greater than 1:32.

There are a number of case reports[28,29,30] which suggest the potential clinical importance of tolerant isolates. Denny and colleagues[31] have provided the most convincing evidence, in terms of staphylococcal infections, that tolerance can be an important factor in the clinical failure of antimicrobial therapy. In one group of ten patients, these investigators reported that initial treatment for serious *Staphylococcus aureus* infections (predominantly endocarditis) with antibiotics to which the isolates were tolerant resulted in a mortality of 40% which was significantly higher than that in a second group of patients (0% mortality) who received bactericidal agents (P = 0.043).

Even in the surviving patients in the first group, three of six required surgery to eradicate the infection (one patient had recurrent infection despite both surgery and one month of antimicrobial therapy) versus only two of ten of patients in the second group who required surgery; none had recurrent infection. Similarly, Rajashekaraiah[32] has reported that infection caused by a tolerant strain of *S. aureus* adversely influences the outcome of therapy for staphylococcal endocarditis.

1.3.1.4 Phenotypic Resistance

It is possible for microorganisms to develop resistance during the actual performance of a susceptibility test. Most often, this is a phenotypic resistance[33,34] which is an inherent characteristic of the microorganism. Tests for bactericidal activity are particularly likely to select phenotypically resistant strains from the population; unlike persisters, these survivors will demonstrate an increase in resistance when retested.

An example of the development of such resistance can be seen with aminoglycosides and gram-negative bacilli. Recent studies[35-41] have shown that the first exposure of gram-negative bacilli to an amino-glycoside results in the down-regulation of bacterial transport of subsequent drug doses and leads to phenotypic resistance. The development of these phenotypically resistant subpopulations has been shown to occur *in vivo*[37,40] and has been reported to be clinically important.[41] Further characterization of these phenotypically resistant isolates indicates an impaired ability to transfer aminoglycosides across the cell membrane due to defects in the cytoplasmic transport system caused by inefficient energy generation.[42,43]

Another example of the development of phenotypic resistance is seen with β-lactam agents and certain gram-negative bacilli, such as *P. aeruginosa*. The ability of these microorganisms to increase the amount of periplasmic β-lactamase while limiting the influx of β-lactam agents through porin channels is an important resistance mechanism.[44]

1.3.2 Technical Factors

1.3.2.1 Growth Phase of Inoculum

The most frequent technical pitfall encountered in microbiology laboratories performing bactericidal testing is the use of stationary-phase cultures. The rate of loss of viability of bacterial cells following exposure to most antibiotics with an especially marked effect on cell-wall-active agents is a direct function of bacterial growth prior to the addition of the agent.[17,45] Aminoglycosides and quinolones, however, are much less affected by the use of slowly growing cultures.[45] The use of stationary phase cultures, such as recently inoculated cultures growing for periods >8 hours, will include an increased number of dormant cells which are not as susceptible to the agent and therefore cause diminished killing rates.

In addition, the use of lag phase cultures (e.g., cultures which have recently undergone a change in test condition such as temperature shift or change from agar to broth medium) will also include cells less reactive to the agent and therefore cause less reliable killing endpoints (see Figure 1). If tolerance or killing rates are being determined, then logarithmically growing rather than stationary phase cultures must be used. This need for actively growing cultures has been stressed by many investigators.[46-50]

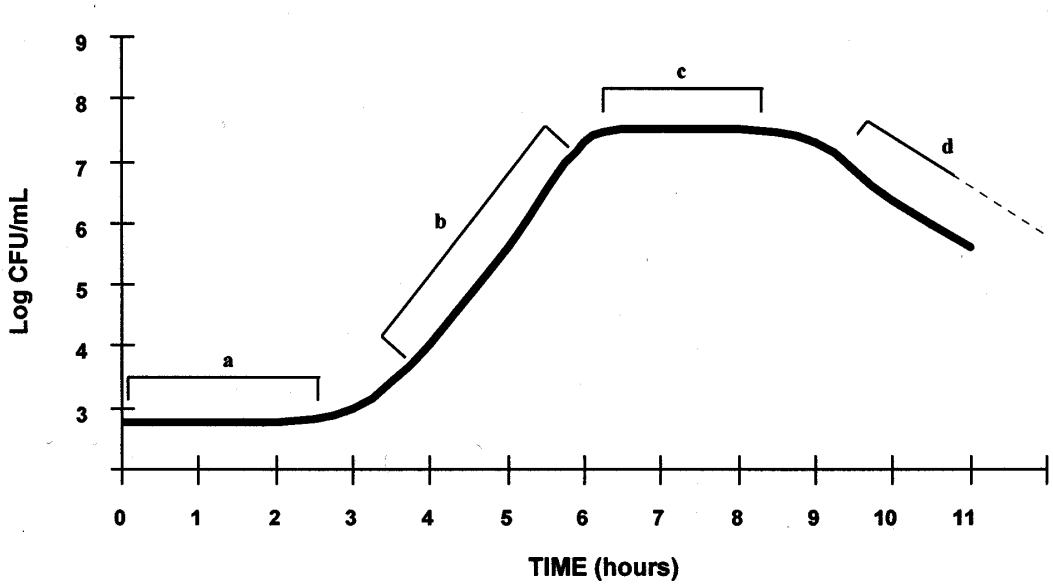

Figure 1. Bacterial Growth Curve. Growth curve of typical bactericidal culture as measured by viable count. a: Lag phase; b: logarithmic phase; c: stationary phase; d: death phase

1.3.2.2 Size of Inoculum

The inoculum effect in susceptibility testing is well known.[51] Frequently, this effect may be attributed to the increased effect of antibiotic inactivating enzymes, such as β-lactamase.[52]

There is, however, another important effect associated with an increased inoculum; this is tolerance. Tolerance appears to increase when large inocula are used and decreases with small inocula. For example, bacteria are killed rapidly when either log- or stationary-phase cultures are diluted to low inocula (10^4), but log-phase cultures concentrated to high inocula (10^7 to 10^8) are killed more rapidly than are stationary phases.

Finally, an increasing inoculum size in log-phase cultures is approaching the stationary growth phase and hence these organisms may be killed more slowly. Thus, both inoculum size and growth phase may independently increase the rate of killing. The inoculum size is considered the single most important variable in susceptibility testing. The inoculum size is critical; variations in density of inoculum may alter then endpoints by one or more dilutions, and therefore the preparation of a standardized inoculum is critical. NCCLS has recommended a final inoculum size of 5 x 10^5 CFU/mL for susceptibility testing of aerobic bacteria in broth.[2]

Because of the importance of the inoculum, great care should be taken in its preparation. Inocula from logarithmically growing organisms (see Section 2.5) should be prepared in a shaker-incubator and flask or beaker, whenever possible, in order to promote uniformity of growth. While the interim inoculum size estimation can be conducted by comparison of actively growing cultures with McFarland turbidity standards for inoculation of drug-containing broths on the day of testing, the final inoculum size approximately 5 x 10^5

CFU/mL should be confirmed by a colony count for interpretation of killing endpoints, the following day.

1.3.2.3 Insufficient Contact

Test organisms may have insufficient contact with the antimicrobial agent. This is most often due to the adherence of viable microorganisms to the culture vessel surface above the meniscus.[47,50,53,54] Such adherence is more likely with plastic test tubes than with acid-treated borosilicate glassware or with plastic microdilution trays. Vortexing at 20 hours and again at 24 hours for tests done in test tubes or continuous shaking for tests done in flasks or bottles allows better contact between all cells and the antimicrobial agent.

1.3.2.4 Antibiotic Carryover

The transfer of a quantitative volume of broth for the count of survivors can be complicated by antibiotic carryover.[55] This problem occurs mainly at higher concentrations (> 16 x MIC). Antibiotic carryover can be detected by using a swab to streak a sample of the test broth across the surface of a dried agar plate (prepared by incubating the plate for 1 hour at 35 ºC), allowing 20 minutes for antimicrobial absorption into the agar, then cross-streaking the inoculum over the entire surface of the plate, and after 24 hours of incubation, looking for inhibition of colonial growth at the site of the initial streak.[56]

Antibiotic carryover can be eliminated by inactivating the antibiotic on the subculture plate. This is easily done for β-lactam agents by flooding the plate with 1 mL of a β-lactamase mixture containing both Types I and II β-lactamases from *Bacillus cereus*.

Alternatively, the sample for subculture can be diluted or washed. Washing the cells can be done by trapping the bacteria in the sample being subcultured on a 0.22 mm-pore size filter, washing them with normal saline, and then immediately suspending them in saline to their original sample volume before they are quantitatively subcultured onto agar. Carryover can be minimized, however, if the inocula are allowed to dry (i.e., the drug allowed to diffuse throughout the agar before streaking the sample of isolated colonies).

1.3.2.5 Volume Transferred

The volume transferred for the count of survivors should be such that after the defined percentage of killing (99.9%), at least ten colonies are present to be counted after incubation. For a 99.9% ideally killing of a final inoculum of 5 x 10^5 CFU/mL, approximately 100 viable cells will remain. A subculture of 10 to 100 µL is transferred. Transferring more than 100 µL is not recommended because of drug carryover. Smaller transfer volumes (less than 10 µL) can result in too few colonies because of pipetting error and intrinsic sampling error due to the Poisson distribution of sample response (not all organisms can be assumed to be equally distributed in a broth prior to sampling[57,58]).

Finally, the volume transferred for the count of survivors must be accurately quantitated. This requires the use of a calibrated micropipette or calibrated stainless steel prongs on a multipoint inoculator. If stainless steel prongs on a multipoint inoculator are used for transfer, the volume delivered by the prong must be confirmed by methods such as the Evan's blue method used for calibrating quantitative loops. Disposable plastic multipoint inoculators have been found to be unsuitable because they do not consistently deliver the prescribed amount.[58] Quantitative loops, even if calibrated, have been found to be unreliable in bactericidal testing because of poor accuracy (± 50%).[59]

1.3.2.6 Choice of Media

The choice of media also needs to be considered. The media ordinarily used for bactericidal testing have little in common with the serum and interstitial fluid of the patient. Among the variables in media and serum known to affect the bactericidal activity of certain antimicrobial agents and microorgan-isms are proteins,[60-64] pH,[65] phosphates,[66] osmolality and salt concentrations,[66,67] and divalent cations.[68] Interaction among the variables, moreover, is complex.

Many types of broth media are used for susceptibility testing, causing markedly different results. Examples of the importance of bactericidal activity being determined in appropriate media can be seen both *in vitro*[69] and *in vivo*.[70] One solution to medium-dependent effects would be to use human serum as the broth medium.

Human serum has disadvantages, however, including instability of pH,[71] risk of transmission of hepatitis B virus, HIV, or other blood-borne infectious agents, inherent antibacterial activity,[72,73] cost and lack of availability, poor support of growth of some organisms, and irreversible binding or increased degradation for some antimicrobial agents.[74,75]

Cation adjusted Mueller-Hinton broth (CAMHB) has been recommended by NCCLS as the medium for broth dilution susceptibility testing.[2] CAMHB more closely resembles serum in pH, osmolality, Na^+, K^+, and Cl^- than do other common broths. CAMHB also demonstrates fairly good batch-to-batch reproducibility, is low in sulfonamide and tetracycline inhibitors, and supports growth of most rapidly growing pathogens.

Additional supplements can be added to this medium to grow such fastidious organisms as *Haemophilus* species and certain streptococci. Calcium and magnesium ion concentrations are especially important when testing amino-glycosides against *Pseudomonas aeruginosa*. In addition, much information about tests performed with CAMHB is available; if another broth were to be selected, most of the previous studies would have to be repeated. The use of salt-containing media must be avoided when testing staphylococci and β-lactam agents.

CAMHB has been combined with human serum in a 1:1 ratio (CAMHB/HS) when testing highly protein-bound agents, e.g., ceftriaxone and nafcillin. The effect of using serum to diminish antimicrobial activity as probably occurs *in vivo* has been well documented for situations in which highly protein-bound antimicrobial agents are used.[64,76-81]

Data are also available to suggest that human serum may alter the results of bactericidal testing for such microorganisms as enterococci.[69] That the use of human serum in bactericidal testing may be important is suggested by a number of studies.[70-82] MICs (and MBCs) of most antimicrobial agents determined with and without human serum against strains of bacteria commonly used as quality control organisms in susceptibility testing[83] are available for quality control programs for those laboratories using CAMHB/HS (see Table 3).

Because of the vagaries of human serum, animal serum has been substituted for human serum in some laboratories. However, the binding of antimicrobial agents by human serum differs from the binding by serum from other animal species including canine, equine, ovine, lapine, and bovine species.[84] Therefore, only pooled human sera or an ultrafiltrate thereof should be used.[85]

Lastly, some microorganisms may not grow adequately in either human serum or CAMHB/HS; in that situation, any alternative broth medium that can support growth of the microorganism can be used.

1.4 Clinical Relevance

Many investigators have used the MBC in an attempt to correlate *in vitro* data with *in vivo* treatment results. Unfortunately, the definition of the MBC (≥ 99.9% killing of the final inoculum after only a single time point, e.g., 24 hours of incubation) is arbitrary and separates the bacteria into two populations, a segregation which might not have biological relevance. The arbitrary threshold of 99.9% killing effect is also generally applied to timed-kill curves; however, the dynamics of the bactericidal activity over time may provide useful information for comparative studies of different agents or studies of the same agent under different test conditions. The determination of the MBC, moreover, is so subject to variables[50,56,86] that the clinical relevance is nearly impossible to assess.[87] It is not surprising, then, to find there are very few published clinical studies that clearly demonstrate a correlation of MBCs with clinical outcome despite the acknowledged need for bactericidal activity for certain infections.

Bamberger et al.[88] found in an infected chamber model in rabbits that *in vitro* synergy was predictive of *in vivo* success if the free antibiotic concentration at the site of infection exceeded the MBC of the aminoglycoside when tested alone and exceeded the MBC of the β-lactam agent when tested in combination with the aminoglycoside. Standiford et al.[89] found that four of five neutropenic patients with gram-negative bacteremia in whom empiric combination therapy failed had no serum bactericidal titers at trough levels. This is in distinct contrast to the 21 patients who survived: all having measurable serum activity

(geometric mean titer of 1:15) at trough levels.

Although the clinical outcome of most infections depends largely on host factors, there are infections such as bacterial meningitis, which carry a 100% mortality rate unless appropriate antimicrobial agents are used. The rapidity for sterilization of the CSF in meningitis is important. McCracken[90] found prompt clinical improvement in five of six infants with sterile CSF on the first follow-up culture, whereas 12 of 13 infants with persistently positive cultures continued to manifest seizures, abnormal reflexes, fever, and poor feeding until the CSF was sterilized. The use of ampicillin/gentamicin in neonates with gram-negative bacillary meningitis has an average time to CSF sterilization of three to five days.[90] The use of ampicillin/gentamicin in 17 neonates with gram-negative bacillary meningitis enrolled in the Neonatal Meningitis Cooperative Study[91] was associated with a mortality rate of 30%,despite the fact that these infants received intrathecal gentamicin as well as parenteral intravenous therapy. The newer cephalosporins, although they do not appear to penetrate into the CSF any greater than older agents,[92] have greater activity which may allow more rapid sterilization of the CSF. Jacobs[93] studied cefotaxime in 18 infants with gram-negative bacillary meningitis and found that 17 (94.4%) survived. Follow-up cerebro-spinal fluid cultures at 24 hours were sterile in all patients.

There is additional clinical evidence that the greater rate of bacterial killing by a *third generation* cephalosporin as compared with a *second-generation* cephalosporin is clinically important. It is clear from three large studies[94-96] that delayed sterilization is more likely with a penicillin[94] or a *second generation* cephalosporin.[95,96] In addition, the slow bactericidal rate of vancomycin has been suggested as a possible reason for the delayed therapeutic response seen in patients with MRSA endocarditis, with organisms thought to be in the lag phase.[96]

Finally, the lack of rapid bactericidal activity of cephalosporins may be related to treatment failures of gram-negative bacillary meningitis.[97,98] When isolates from meningitis cases which were successfully treated with moxalactam were compared to isolates where moxalactam therapy failed, these isolates had identical MICs and MBCs but the killing curves at different multiples of the MIC were markedly different, with the isolates from moxalactam failures exhibiting a marked decrease in the killing rate.[98]

These studies are important because they demonstrate the importance of bactericidal activity for certain infections. Because of this need for bactericidal activity, bactericidal testing is, on occasion, needed and the methodology should be "standardized." These studies also serve to point out the potential usefulness of kill-kinetic methodology in which sequential samples are subcultured over time from a logarithmically-growing culture to determine the rate of killing of microorganisms (see Section 2.3). Further studies are needed to evaluate broth killing curves and to establish the significance and reproducibility of kill-kinetic methodology as a laboratory measurement of bactericidal activity that might eventually replace the MBC test for those instances when bactericidal testing is indicated.

However, the increasing numbers of immunocompromised patients as well as patients with endocarditis will result in requests for testing. Most immunocompetent hosts with bacterial infections do not require antimicrobial therapy in which there is bactericidal activity.

Testing a specific clinical isolate and subsequent interpretation of results should be done only in relation to known clinical evidence of responsiveness or lack thereof. For example, a patient with endocarditis caused by *Enterococcus faecalis* who is changed from ampicillin-gentamicin therapy (despite a favorable clinical response) to alternative treatment with a cephalosporin based on bactericidal testing, may receive inappropriate therapy due to the lack of appreciation of certain technical factors[99] associated with the susceptibility testing of this microorganism.

On the other hand, lack of response in a patient with enterococcal endocarditis could justify a bactericidal test to ensure that the isolate was killed by the agents being used. Bactericidal tests should not be given excessive credibility nor should they be used inappropriately.

2 Test Methods for Determining Bactericidal Activity

2.1 Preparation of Antimicrobial Solutions

Antimicrobial solutions should be prepared with standard powders of known potencies. These are available by request from manufacturers, or can be purchased from the U.S. Pharmacopeia (12601 Twinbrook Parkway, Rockville, Maryland 20852). Standard powders are dissolved according to directions supplied by their manufacturers. It is convenient to prepare initial concentrations that are ten times the desired final concentrations. These can be kept in 1-mL aliquots at -70 ºC for periods up to six months. A new aliquot should be thawed for each day's use. (For additional details refer to the most current version of NCCLS document M7—*Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically* and the Appendix by Dr. Anhalt in the ASM *Manual of Clinical Microbiology.*[100])

2.2 Broth Medium

2.2.1 Mueller-Hinton and Human Serum

The recommended broth is Mueller-Hinton broth (MHB). Especially when testing *Pseudomonas aeruginosa,* the divalent cations Ca^{++} and Mg^{++} must be adjusted as needed to approach free physiologic concentrations and to conform to NCCLS MIC performance standards.[2]

Cation-adjusted MHB (CAMHB) is available from several manufacturers. CAMHB can be used with human serum in a 1:1 ratio (CAMHB/HS) if desired. The use of human serum depends upon the antimicrobial agent being tested (e.g., highly protein-bound agents i.e., >90%), the bacteria being tested (e.g., enterococcus), and the type of bactericidal test being done (e.g., serum bactericidal test). Finally, the choice of broth may be altered for research purposes or in order to grow fastidious bacteria. Addition of NaCl to the manufacturer's formulation of broths is not recommended when testing staphylococci and β-lactam antibiotics because it will render the organisms difficult, if not impossible, to kill. Differences in test results due to the choice of broth must be considered in interpreting results.

2.2.2 Routine Monitoring

The performance and chemical characteristics of both Mueller-Hinton broth and human serum must be routinely monitored.[2] The pH of each batch of Mueller-Hinton broth should be checked with a pH meter when the medium is prepared; it should be between 7.2 and 7.4. The MIC characteristic of each batch of broth should be evaluated using a standard set of quality control organisms with an antibiotic from each major class.[2]

Pooled human serum can be obtained commercially from several sources. It must be quality controlled as rigorously as any other medium used in a clinical microbiology laboratory. For the safety of laboratory personnel, it should be screened for hepatitis B virus antigen and for antibodies to HIV. The pooled human serum may be heated to 56 ºC for 1 hour upon receipt in order to inactivate HIV and to inactivate complement. Next, the serum should be adjusted with 0.1 N NaOH or 0.1 N HCl to pH 7.3 to 7.4 at 25 ºC, and clarified by prefiltering with a 0.80-micron filter. Finally, the sera should be filter-sterilized using a 0.2-micron filter.

Pooled human serum may contain substances to neutralize antibiotic agents, e.g., β-lactamases. For this reason, pooled human serum should be tested for the presence of β-lactamase activity. There are a number of commercial rapid β-lactamase tests available. Any of these except those utilizing PADAC[101] can be used by dropping some of the sera on the test strip and observing for a color change over 30 min. The serum should be screened for nonspecific antimicrobial activity even if stated to be antibiotic-free, by 1) placing 20 µL of the pooled human serum on blank paper disks; 2) placing the disks onto Mueller-Hinton agar which has been seeded with a spore suspension of *Bacillus subtilis* (ATCC® 6633); and 3) looking for zones of inhibition after incubation at 35 ºC for 24 hours. Zone sizes of ≤ 6mm can be ignored. A final quality control measure that can be used if desired is to determine MICs and MBCs of various antimicrobial agents for control strains of bacteria in media supplemented with the serum.

MICs and MBCs of many antimicrobial agents have been determined with and without human serum against strains of bacteria commonly

used for quality control of susceptibility tests.[102] The pooled human serum should be reheated to 56 ºC for 30 minutes just before use in order to ensure that complement is inactivated. The serum may be stored at -20 ºC or less until needed.

Despite the theoretical and proven importance of using human serum as the diluent, there is a multitude of problems associated with the use of pooled human serum in the clinical microbiology laboratory. This problem can be avoided simply by using an ultrafiltrate of human serum. This separates the free drug present and allows the use of Mueller-Hinton broth as the diluent. The use of an ultrafiltrate in the measurement of serum bactericidal activity avoids the disadvantages (e.g., infectious hazard and contribution of antimicrobial activity) associated with the use of normal pooled human serum for the diluent. Ultrafiltration of serum is easily accomplished by centrifugation at 25 ºC for 30 minutes at 1000 x g using a commercially available filtration device. Sterilization of the ultrafiltrate is done by filtration through a 0.2 µ-filter.[101]

2.3 Time-Kill Method

2.3.1 Background

The determination of the killing rate of a bacterial isolate by an antimicrobial agent (or combination of agents) has been applied widely to the evaluation of new drugs. Using such a killing curve for guiding chemotherapy in an individual patient is rarely done.

There is interest in time-kill methods for a number of reasons.

- Although several experimental studies, as measured by MBCs, have shown poor correlation of bactericidal activity with cure of endocarditis,[103,104] most such studies have shown that the time-kill curve technique has the best correlation with cure.[105,106]
- Time-kill studies are one of the most reliable means of determining tolerance.[16,26]
- The time-kill method is useful for determining synergy or antagonism between two (or more) antimicrobial agents.[107,108]

Studies have shown that there are important differences between the results of synergy testing with killing curves and the results using the checkerboard technique[108-112] and that the time-kill curve method correlates best with cure in animal models.[105,108,113,114] This is not surprising because checkerboard results are assessed only at one time-point whereas the killing curves measure changes over time. The slope of killing curves done with different concentrations of a single agent enables one to compare the rates of decline in the survivor count. This actual rate of killing may be more important than the concentration at which 99.9% killing of the final inoculum occurs.

When killing curves are used to assess an antimicrobial agent, testing at several multiples of the MIC is recommended. At intervals (usually 0, 4, 8, 10 to 12, and 24 hours of incubation) colonies are counted, and the results are charted on semilog paper with the survivor colony count on the ordinate in logarithmic scale and the time on the abscissa in arithmetic scale. (Results can be converted to $\log_{10}$ and regular graph paper used, if desired.)

Over time, colony counts may increase after an initial decrease. This can be due to the selection of resistant mutants, inactivation of the antimicrobial agent, or regrowth of susceptible bacterial cells which have escaped the antimicrobial activity by adhering to the wall of the culture vessel.[50,53,54] The clinical importance of such regrowth (should it occur) is unclear, particularly if it occurs after the time equivalent to the usual dosing interval of the antimicrobial agent being tested. Several factors should be considered:

- The time at which the regrowth occurs
- The dosing of the antibiotic in the clinical setting
- The type of bacteria
- The antibiotic itself.

When regrowth occurs, it is useful to determine the MICs of survivors in order to see if a resistant organism was selected from the population tested. Inactivation of the antibiotic can be determined by appropriate assays at time 0 and at 24 hours. A simple method for checking for inactivation of the antibiotic is to

use a sample of broth (filter-sterilized) at time 0 and at 24 hours in order to perform MICs with an appropriate ATCC reference strain. If the MIC at 24 hours is markedly higher than that at 0 hours, the antibiotic has been inactivated. If regrowth cannot be explained by either drug inactivation or emergence of a resistant subpopulation, repetition of the test would be helpful with interpretation of results.

Methods for interpretation of kill-kinetic studies may vary. Bactericidal activity as defined by 99.9% killing of the final inoculum can be determined from time-kill curves by noting the presence or absence of a $\geq$ 3-$\log_{10}$ decrease in CFU/mL. Synergy is defined as a $\geq$ 2-$\log_{10}$ decrease in CFU/mL between the combination and its most active constituent after 24 hours with the less active component being tested at an ineffective concentration. Some investigators consider the slope from four to eight hours as the most important factor for antibiotics that are administered frequently.[98,106]

A new approach uses serum taken from a subject to whom an antibiotic has been administered and measures the rate of serum killing (serum bactericidal rate).[115,116] Linear regression analysis is used on the resultant curves to determine the serum bactericidal rate (defined as the slope of the regression line with units being the change in the $\log_{10}$ CFU/mL per hour of exposure to the agent). For purposes of comparison, the more negative the slope, the faster the rate of bactericidal activity. Linear regression can be used to define the slopes for time-kill curves.

Another method for comparing time-kill curve is to calculate the mean percent killed at various times. If the time-kill method is chosen, it is important for the user to determine the intralaboratory reproducibility of colony counts ($\log_{10}$ CFU/mL) which may impact the interpretation of results.

2.3.2 Procedure

The time-kill curve method is done in glass tubes (flasks or beakers, whenever possible) each containing $\geq$ 10 mL of MHB or suitable broth and the chosen concentrations of antimicrobial agent(s) to be tested. Flasks, beakers, or bottles allow a larger volume of broth to be tested which results in a greater challenge to the antimicrobial agent because of the absolute number of organisms and optimization of growth, and hence, kill rate. One tube or flask without antibiotic is used as a growth control. The inoculum is prepared according to methods used in other types of susceptibility testing.[2] (Section 2.5 contains details for this step.) If test tubes are used, the inoculum should be added in a manner that avoids any splashing on the inside of the test tube above the meniscus.[50] Tubes should be vortexed before sampling in order to resuspend bacteria adhering to the wall. (Determination of colony counts is described in Section 3.1.)

2.4 Dilution Methods

Bactericidal activity also can be determined by dilution methods. Broth dilution MBCs represent an artificial index of bactericidal activity, as defined by an arbitrarily selected 24-hour time point of antimicrobial action and an arbitrarily selected 99.9% killing of the initial inoculum (0.1% survivors). Both broth dilution[57] and agar dilution[117-121] methods can be used. The agar dilution method immobilizes the final inoculum in an agar-gel matrix and depends upon inactivation of the antimicrobial agent in order to determine regrowth of viable CFU after a specific period of incubation. To date, the agar dilution method has been used only for evaluating the bactericidal activity of β-lactam agents[117-119] which can easily be inactivated by adding β-lactamases. By inactivating the β-lactam agent at various times (e.g., 3, 6, 8, 12 hours), time-kill curves can be obtained with the agar dilution method.

The advantage of the agar dilution plate count method for the determination of bactericidal activity is that it minimizes the introduction of spurious results due to technical factors. Because the bacteria are immobilized in an agar-gel matrix, both throughout the time of bacterial exposure to the antibiotic and during the time for regrowth of viable CFU after inactivation of the agent, the technical factors (such as sequestration of the bacteria on container walls above the broth surface, the use of stationary-phase inoculum, and the carryover of inhibitory amounts of antimicrobial agents onto subculture plates) are minimized.

Broth dilution MBCs are determined by first performing the standard broth dilution technique for MICs.[2] Then, sampling of those dilutions having no visible growth can be done with a calibrated device to determine the concentration

at which 99.9% of the final inoculum is killed. Like MICs, MBCs can be determined with a macrodilution method (1 to 2 mL in each test tube) or a microdilution method (0.1 mL in each well). A bactericidal endpoint of 99.9% killing of the final inoculum is more difficult to assess with the microdilution method.[58] However, the microdilution method especially when testing β-lactam agents and staphylococci is the recommended method of choice for bactericidal testing due to the common usage of this method and reports of greater reproducibility compared to the macrodilution method.

The procedures described for the microdilution and macrodilution method usually involve twofold dilutions of the antimicrobial agent. A disadvantage of twofold dilutions is that there are larger absolute differences at the higher dilutions. The advantage, of course, is that they are easy to perform.

Using intermediate dilutions is an alternative approach that eliminates the wide gaps at higher concentrations. In this method, dilutions are calculated to yield small incremental arithmetic dilutions at half-dilution intervals.

However, this is more technically difficult to do. Moreover, the clinical relevance of small incremental dilutions remains to be demonstrated. Twofold dilutions are recommended as the most practical method for a clinical laboratory.

2.4.1 Agar Dilution Plate Count Method

The agar dilution plate count method is performed by first preparing Mueller-Hinton agar plates containing twofold dilution concentrations of the desired β-lactam agent.[2] Each plate of a dilution panel is inoculated by pipetting 0.05 mL (5 x 10^5 CFU per plate) of a standardized inoculum preparation onto the agar surface. Streak immediately with a bacteriologic loop or pipette tip to disperse the inoculum in at least three opposing directions. The streaked plates are allowed to dry for approximately 15 minutes, at which time they are overlaid with 10 mL of molten (48°C) Mueller-Hinton agar containing an analogous concentration of the β-lactam agent.

2.4.2 Macrodilution Method

The macrodilution method should be performed in sterile 13 x 100-mm acid-treated borosilicate glass test tubes. (Bacteria adhere to the walls of plastic tubes.) The following basic procedure is performed for aerobic bacteria as described for MIC determinations.[2]

(1) Add 1.0 mL of CAMHB or heat-inactivated pooled human serum to tubes 2 through 10.

(2) Use the same broth used in tubes 2 through 9 to prepare the antibiotic solution.

(3) Add 1 mL of the antibiotic solution to each of the first two tubes.

(4) Make serial twofold dilutions beginning with the second tube and continuing through the ninth tube, leaving an intermediate volume of 1.0 mL in each tube. (**Use a separate pipette for each tube**.) Dilutions are made by withdrawing 1.0 mL from the tube containing 2.0 mL and transferring this to the next tube which is then mixed on a vortex mixer before the next step is repeated. Discard 1.0 mL from the ninth (last) tube.

(5) After completing the dilutions, add 1.0 mL of MHB to each tube in order to yield a final volume of 2.0 mL in each tube; and if pooled human serum is used, to yield a 1:1 ratio of pooled human serum and Mueller-Hinton broth.

The tenth tube serves as a control for growth of the organism.

2.4.3 Microdilution Method

The microdilution method is performed in sterile plastic microdilution trays that have round or conical bottom wells, each containing a final volume of 0.1 mL of broth. Adherence of organisms to the sides of the wells has not been found to be a problem unlike that seen with the use of plastic test tubes for the macrodilution method. The microdilution method is simple and more efficient to perform and reduces the amount of broth required. The basic procedure for aerobic bacteria is performed as described for MIC determi-nations.[2]

Commercial or in-house previously prepared MIC plates may be used or they may be prepared as needed by the following method:

(1) Add 0.05 mL of broth (CAMHB or pooled human serum) to each well of column 2 through 10 in each row. The same broth as used in wells 2 through 10 is used to prepare the antibiotic-containing solution.

(2) Add 0.05 mL of this solution to each of the first two wells.

(3) Make serial twofold dilutions of the antibiotic from column 2 through column 9 with a semiautomatic microdilution device employing 0.05-mL microdiluters, transferring and moving 0.05 mL to each well. Discard 0.05 mL from the ninth well.

The tenth well is a positive growth control containing 0.1 mL of the broth being used with no antibiotic.

2.5 Preparing Inoculum

If the test isolate was stored (e.g., at -20 ºC or -50 ºC) it is advisable to subculture the isolate three times before testing to ensure that the organism has an optimal growth and metabolic status before the drug exposure. Preparation of the inoculum is similar to other methods used for susceptibility testing.[2]

(1) To address the possibility of heterogeneously distributed resistance among colonies, touch lightly between 5 and 30 colonies of a single morphological type from a 16- to 24-hour agar plate containing a nonselective culture medium and inoculate them into a tube containing 5.0 mL of prewarmed (35 ºC) broth (Mueller Hinton or trypticase soy broth). For gram-negative bacteria, fewer colonies (five colonies) are needed than for gram-positive bacteria.

(2) Incubate this bacterial suspension at 35 ºC until its visibly turbid, e.g., up to 6 hours for staphylococci and < 6 hours for gram-negative rods. It is recommended that this log-phase inoculum be prepared in a flask, beaker, or bottle and incubated with a shaker-incubator to promote uniformity and optimization of growth.

(3) Adjust the turbidity of the actively growing broth culture (logarithmic phase) to obtain a turbidity visually comparable to that of a 0.5 McFarland turbidity standard.[2] Dilute the adjusted culture in broth (macrodilution method) or 0.9% saline (microdilution method) so that after inoculation, each tube or well contains approximately 5 x 10^5 CFU/mL. The number of CFU/mL in the broth medium just prior to incubation, i.e., the final inoculum, is estimated by comparison to a McFarland turbidity standard and then used to inoculate antibiotic solutions. However, the colony count of this final inoculum must be determined by serial dilution in saline and subculture to medium and overnight incubation to allow interpretation of killing endpoints.

The dilution procedure to obtain this final inoculum varies according to the method and must be calculated for each system. The exact inoculum volume delivered to the tubes or wells must be known before this calculation can be done. For example, if the volume of medium in the tube is 2.0 mL, and the inoculum is 0.1 mL, the adjusted culture (1.5 x 10^8 CFU/mL) must be diluted 1:20 with broth to yield 7.5 x 10^6 CFU/mL. When 0.1 mL of this latter suspension is inoculated into 2.0 mL of broth, the final inoculum of bacteria will be approximately 4 x 10^5 CFU/mL. Because the minimal bactericidal test measures 99.9% killing of the final inoculum, a number of aliquots of final inoculum are prepared at 35 ºC if the tube or small flask method is being employed. The first aliquot is used to measure the inoculum size by serial dilution in saline and subculture to solid medium so that on the next day the killing endpoint can be properly determined. Other identical aliquots are used to inoculate antibiotic solutions.

The viable colonies in the inoculum should be counted in order to verify the final inoculum size. (This can easily be done by using the growth control test tube or a second growth control well in the microdilution plate.) This is done by using a micropipette to deliver 0.1 mL of each of a 10^{-2}, 10^{-3}, and 10^{-4} dilution of the final inoculum in saline onto the surfaces of agar plates[36] and then spreading this over the surface. After overnight incubation, the plate showing 20 to 200 colonies is used to calculate the final inoculum size. For example, if there are 40 colonies on the plate inoculated with 0.1 mL

of a 1:1000 dilution (10-4 final dilution), then the inoculum contained (40 x 10^4 or) 4 x 10^5 CFU/mL.

2.5.1 Inoculating Broth

2.5.1.1 Macrodilution Method

(1) Prepare the dilutions of the antimicrobial agent before adjusting the inoculum.

(2) Within 15 minutes after the actively growing inoculum has been standardized, add 0.1 mL of the adjusted inoculum to each tube in the dilution series with a pipettor to release *the inocula beneath the surface* of antimicrobial-containing broth.

(3) Mix (without creating air bubbles or splashing the sides of the tubes) by hand or vortex at low speed.

(4) Do not shake or agitate the tubes further.

2.5.1.2 Microdilution Method

As in the macrodilution method, the dilutions should be made before adjusting the inoculum. The inoculum then should be diluted and used to inoculate the broth within 15 minutes after the inoculum is standardized.

(1) Prepare or thaw the plates before adjusting the inoculum.

(2) Within 15 minutes after the actively growing inoculum has been standardized, dilute the inoculum so as to achieve the appropriate final concentrate.

(a) If the volume of the inoculum exceeds 10% of the well volume, the diluting effect of the inoculum on the antimicrobial must be taken into account. If a 0.05-mL pipette dropper is used to add the inoculum to the 0.05-mL broth-containing wells, the antimicrobial agent dilution is 1:2 which results in a final range of dilutions that is half the initial concentration in each well.

(b) An alternate way to inoculate the wells is to first add 0.05 mL of broth to each well after the dilutions have been made and then add 0.01 mL of inoculum using a calibrated multipoint inoculator. In this case, the number of organisms in the seed tray should be 5 x 10^6 CFU/mL and the actual inoculum in a growth control well must be determined immediately after inoculation. To prevent evaporation during incubation, each tray should be sealed in a plastic bag, with plastic tape, or with a tight-fitting plastic cover before incubation.

2.5.2 Incubation

For all test methods, the plates, tubes, or trays should be incubated at 35 ºC in air or with CO_2, if required for growth of the patient's isolate. Macrodilution tubes should be incubated 20 hours, vortexed and reincubated at 20 hours, then vortexed and reincubated again before sampling at 24 hours. Microdilution trays should be incubated for a total of 24 hours and should be shaken at 20 hours but *not* before sampling at 24 hours. To maintain the same incubation temperature for all cultures, microdilution trays should not be stacked more than four high.

3 Determining Endpoints

3.1 Time-Kill Method

Sampling for colony counts is done by removing 0.5-mL samples of the broth at specified times. The times include zero hours, and usually 4, 8, 10 to 12, and 24 hours. These samples are serially diluted in test tubes containing 4.5 mL of sterile saline (0.9% NaCl) to produce 10-fold dilutions (10^{-1},10^{-2},10^{-3}, 10^{-4}). There are a number of methods for deter-mining the CFU/mL from these dilutions. Samples (at least 10 µL but no more than 100 µL) can be removed from the serial dilutions, pipetted onto an agar plate, streaked, and cross-streaked 10 to 20 minutes later.[56] Alternatively, 1-mL samples of the serial dilutions can be incorporated into agar pour plates. Yet another method uses 20- to 50-µL samples which are dropped on each of five spots onto warmed (35 ºC for 1 hour) agar plates and allowed to absorb without streaking.[121] Whichever method is used, the minimal, accurately detectable number of CFU/mL must be determined by serial dilutions with a known inoculum.

When higher concentrations (≥4 x MIC) of the antimicrobial agent(s) (e.g., β-lactam agents) are

tested, drug carry-over can be a problem. It may be a problem for drugs with an inoculum effect, even at 1 x MIC. Serial dilutions (10^{-1}, 10^{-2},10^{-3},10^{-4}) in saline may minimize this problem. Drying the inoculum on the agar surface before streaking can also reduce the antibiotic carry-over. Potential problems with drug carry-over can be determined by initially streaking a 100-µL sample of the test organism across the entire surface of a warm agar plate. After 10 to 20 minutes to allow antimicrobial absorption into the agar, the plate is then cross-streaked over the entire surface. Antimicrobial carry-over can be detected by the inhibition of colonial growth at the site of the initial streak.[56] If antibiotic carry-over is a problem, the cells can be washed after sampling from the broth culture.[97] For β-lactam agents or aminoglycosides, additives such as β-lactamase or NaCl, respectively, can be used to prevent the effect of drug carry-over.

Colonies are counted after 24 to 48 hours of incubation. Prolonged incubation (48 hours) facilitates counting colonies, as the colonies are larger. With certain organism/antibiotic combinations, the resultant colonies may be changed (e.g., dwarf size) and not readily detected. Prolonged incubation of plates before colonies are counted may be more accurate. A magnifying lens can facilitate colony counts when plates are incubated for 24 hours.

The results of kill-kinetic determinations can be shown graphically by plotting $\log_{10}$ CFUs against time. A bactericidal effect can be seen by a $\geq$ 3 $\log_{10}$ (99.9% killing) decrease in CFU at the time specified. If synergy of a combination of agents is being measured, synergy can be defined as a $\geq$ 2 $\log_{10}$ decrease in CFU/mL between the combination and its most active constituent. At least one of the antimicrobial agents must be present at a concentration which does not affect the growth curve of the test organism when used alone.

3.2 Agar Dilution Plate Count Method

After incubation of the agar plate for 24 hours, the MIC can be determined. The MIC is defined as the concentration in the first plate of the ascending concentration series for which the plate count is at least two standard deviations below that representing 0.1% of the final inoculum count. MBCs can be done at any specified time period (e.g., 3, 6, 12, 24 hours). MBCs are done by first inactivating the β-lactam agent by flooding each plate with 1 mL of a β-lactamase mixture containing both Types I and II β-lactamases from *Bacillus cereus*. After application of the β-lactamase solution, the plates are reincubated for 48 hours, at which time colony counts are made to determine persister percentages for each β-lactam concentration. The MBC is defined as the concentration in the first plate of the ascending concentration series for which the plate count is at least two standard deviations below that representing 0.1% of the final inoculum count.

3.3 Minimal Bactericidal Concentrations

The definition of the MBC most often used is 99.9% killing ($\geq$ 3 $\log_{10}$ drop in CFU/mL) of the final inoculum.[32] While the final inoculum size can be estimated by comparison with a McFarland turbidity standard for adjustment of size and introduction of inoculum to the kill test cultures, actual CFU/mL must be determined the next day for use in determining the concentration which achieved 99.9% kill. The volume subcultured is based on this final inoculum size and must be done quantitatively.

Because of inaccuracy due to random variation in the number of bacteria in a small-volume sample, this subculture volume should be large enough to provide an endpoint of at least ten colonies, but not so large that antimicrobial agent is carried over in a concentration high enough to continue inhibition of viable cells.[50]

Methods for determining MBCs which take into account pipetting error and intrinsic sampling variability due to the Poisson distribution of sample response have been described.[57,58] In these techniques, the final inoculum size is determined by a method such as the surface-drop count. Then, the lethal endpoint is determined by a quantitative subculture using calibrated pipettes. Each tube or well showing inhibition of growth at 24 hours is subcultured onto a blood agar plate. Rejection values are determined by a chart which considers the final inoculum size, single or double sampling, pipetting error, and the Poisson distribution of sample responses[57] (see Tables 1 and 2). For example, if the final inoculum is 5 x 10^5 and a single sample is used, the dilution having fewer than 11 colonies is the lethal endpoint (see Table 2).

3.3.1 Macrodilution Endpoints

Macrodilution endpoints should be determined as follows:

(1) Use a logarithmic-phase culture for the final inoculum.

(2) The final inoculum should be 5 x 10^5 CFU/mL (range ≥1 x 10^5 CFU/mL to ≤ 1 x 10^6 CFU/mL).

(3) Confirm the final inoculum (5 x 10^5 CFU/mL) by actual count.

(4) Add the final inoculum to the macrodilution tubes in small volume (0.1 mL) and below the surface with no shaking.

(5) Incubate at 35 ºC.

(6) Gently mix by hand (or vortex at a low speed) at 20 hours and again at 24 hours before sampling.

(7) Use a calibrated pipettor to subculture 0.01 mL from each clear test tube; duplicate subcultures should be done.

(8) Streak the broth subcultured onto an agar plate. Incubate the plates for 24 to 48 hours depending upon the growth needs of the test pathogen. Gram-negative rods, e.g., *E. coli*, probably require only 24 hours of incubation whereas the gram-positive pathogens may require longer incubation periods to ensure adequate growth. Use the number of colonies subsequently grown to determine the bactericidal endpoint based on the final inoculum and the rejection values[57] (see Tables 1 and 2).

3.3.2 Microdilution Endpoints

Microdilution endpoints should be determined as follows:

(1) Use a logarithmic-phase culture for the final inoculum.

(2) The final inoculum should be 5 x 10^5 CFU/mL (5 x 10^4 CFU/well) (range 1 x 10^5 to 1 x 10^6 CFU/mL).

(3) Confirm the final inoculum (5 x 10^5 CFU/mL) by actual count.

(4) Add the final inoculum to the wells in an equal volume (0.05 mL) or using a multipoint inoculator (0.01 mL). Do not shake further.

(5) Incubate at 35 ºC.

(6) Remove 0.01 mL of the well after stirring, and spread the contents of each well over a separate blood agar plate. Duplicate 0.01-mL samples can be done to increase the accuracy. Another approach is to aspirate the entire amount and streak that over a blood agar plate.

(7) If 0.01 mL of the clear wells were subcultured, use the number of colonies subsequently grown to determine the lethal endpoint based on the final inoculum (Tables 1 or 2).

(8) Streak the broth subcultured onto an agar plate. Incubate the plates for 24 to 48 hours depending upon the growth needs of the test pathogen. Gram-negative rods, e.g., *E. coli*, probably require only 24 hours of incubation whereas the gram-positive pathogens may require longer incubation periods to ensure adequate growth. Use the number of colonies subsequently grown to determine the bactericidal endpoint based on the final inoculum and the rejection values[57] (see Tables 1 and 2).

4 Interpretation

Interpretation of time-kill curves and MBCs can be difficult. Kill-kinetic results are most easily interpreted during the first 12 hours. It is suggested to have a comparator drug which is known to be bactericidal against the test pathogen. This minimizes the problem with regrowth. Comparison of isolates from clinical cases with isolates from similar cases where cure was achieved appears to be the most predictive way to evaluate kill-kinetic results.[97,98 A] high inoculum (10^7 CFU/mL) may be needed to evaluate microorganisms producing β-lactamase if β-lactam agents are being tested. MBCs are especially difficult to interpret for certain microorganisms and antibiotics. *S. aureus* and β-lactam agents, in particular, are a problem with respect to interpretation because of poor reproducibility with MBC tests.[56,122] Moreover, the paradoxical effect is often seen with *S. aureus*.

The paradoxical effect is defined as the occurrence of progressively increasing plate counts for at least three consecutive concentrations above the MIC. If this occurs, the concentrations exhibiting the paradoxical effect are usually ignored for *S. aureus*, but are taken into account when dealing with those gram-negative bacteria that can be inducible/constitutive producers of Group 1 β-lactamase (e.g., *Enterobacter, P. aeruginosa*).

5 Quality Control

5.1 Purpose

The quality control program monitors:

- The precision and accuracy of the susceptibility test procedure
- The performance of reagents used in the test
- The individuals who perform the test and read the results.

This goal is best realized by using reference strains selected for their genetic stability and for their usefulness in the method being controlled.

5.2 Method Control

An ATCC strain known to be susceptible to the antimicrobial agent being tested should be tested in the same method being used to evaluate a clinical isolate. Comparison of the results can be done and an estimate of how the clinical isolate will respond to the agent can be made.

5.3 Selecting Reference Strains

Ideal reference strains for quality control of dilution susceptibility methods have MICs (and MBCs) that fall near the midrange of the concentration for all antimicrobials tested. An ideal control strain is inhibited at the fourth dilution of a seven dilution $\log_2$ series, but strains with MICs (and MBCs) at either the third or fifth dilution are acceptable. The reference strains will be used primarily as quality control measures for the media used in bactericidal testing.

5.4 Suggested Quality Control Strains

A full set of quality control strains that have adequate or optimal endpoints for all the commonly used antimicrobial agents is not yet available. A number of strains with useful endpoints, however, have been tested repeatedly and have proven to be stable. In addition, expected MICs of many antimicrobial agents have been documented. We currently recommend the following reference strains for controlling dilution tests:

- *Escherichia coli* ATCC® 25922
- *Pseudomonas aeruginosa* ATCC® 27853
- *Staphylococcus aureus* ATCC® 25923
- *Enterococcus faecalis* ATCC® 29212

5.5 Batch or Lot Control

Records of the lot numbers of all materials and reagents used in these tests should be kept.

5.6 Other Control Procedures

5.6.1 Growth Control

Each macrodilution series and each microdilution tray should include a positive growth control of basal medium without antimicrobial agent to assess viability of the test organisms. The growth control also serves as a turbidity control for reading MIC endpoints.

5.6.2 Purity Control

A sample of the inoculum should be streaked on a suitable agar plate and incubated overnight to detect mixed cultures and to provide freshly isolated colonies in case retesting proves necessary. The growth control can be subcultured at the same time that the MBCs are performed in order to assess purity.

Table 1. Rejection Value and Calculated Sensitivity and Specificity for Each Initial Concentration on the Basis of Duplicate 0.01-mL Samples[a]

Final Inoculum (CFU/mL)	Rejection Value[c] (5% Error (Pipette error plus full sampling error) for Determination of Final Inoculum[b])	Sensitivity[d] (%)	Specificity[d] (%)
1×10^5	4	77	97
2×10^5	8	89	99
3×10^5	15	99	99
4×10^5	20	99	99
5×10^5	25	99	99
6×10^5	29	99	99
7×10^5	33	99	99
8×10^5	38	99	99
9×10^5	42	99	99
1×10^6	47	99	99
2×10^6	91	99	99
3×10^6	136	99	99
4×10^6	182	99	99
5×10^6	227	99	99
6×10^6	273	99	99
7×10^6	318	99	99
8×10^6	364	99	99
9×10^6	409	99	99
1×10^7	455	99	99

[a]When the sum of colonies from duplicate samples was equal to or less than the rejection value, the antibiotic was declared lethal (a 0.999 or greater reduction in the final inoculum).

[b]Based on duplicate samples for the determination of the final inoculum size.

[c]Number of colonies.

[d]Sensitivity and specificity calculated for each specific final inoculum concentration and rejection value.

Table 2. Rejection Value and Calculated Sensitivity and Specificity for Each Initial Inoculum Concentration on the Basis of a Single 0.01-mL Sample[a]

Final Inoculum (CFU/mL)	Rejection Value[c]	Sensitivity[d] (%)	Specificity[d] (%)
	5% Error (Pipette error plus full sampling error) for Determination of Final Inoculum[b]		
1×10^5	3	84	83
2×10^5	4	87	97
3×10^5	6	84	98
4×10^5	8	89	99
5×10^5	11	96	99
6×10^5	15	99	99
7×10^5	17	99	99
8×10^5	20	99	99
9×10^5	23	99	99
1×10^6	25	99	99
2×10^6	47	99	99
3×10^6	68	99	99
4×10^6	91	99	99
5×10^6	113	99	99
6×10^6	136	99	99
7×10^6	159	99	99
8×10^6	182	99	99
9×10^6	204	99	99
1×10^7	227	99	99

[a]When the number of colonies from a single sample was equal to or less than the rejection value, the antibiotic was declared lethal (0.999 or greater reduction in the final inoculum).

[b]Based on a single sample for the determination of the final inoculum size.

[c]Number of colonies.

[d]Sensitivity and specificity calculated for each specific final inoculum concentration and rejection value.

Table 3. Suitable Quality Control Ranges for MICs and MBCs With and Without Human Serum Using ATCC Strains[a]

Staphylococcus aureus ATCC® 25923

	MIC in		MBC in	
Drug	CAMHB[b]	CAMHB/HS[c]	CAMHB[b]	CAMHB/HS[c]
Methicillin	1-4	1-4	1-4	1-4
Oxacillin	0.125-0.5	0.5-2	0.25-1	1-4
Nafcillin	0.125-0.5	0.5-2	0.25-1	1-4
Cephalothin	0.0625-0.25	0.25-1	0.125-0.5	0.5-2
Cefazolin	0.125-0.5	0.5-2	0.125-0.5	1-4
Vancomycin	0.5-2	0.5-2	1-4	1-4

Escherichia coli ATCC® 25922

	MIC in		MBC in	
Drug	CAMHB[b]	CAMHB/HS[c]	CAMHB[b]	CAMHB/HS[c]
Ampicillin	2-8	2-8	4-16	4-16
Piperacillin	1-4	1-4	2-8	2-8
Cephalothin	8-32	16-64	16-64	32-128
Imipenem	0.0625-0.25	0.25-1	0.125-0.5	0.25-1
Gentamicin	0.25-1	1-4	0.25-1	1-4
Amikacin	0.5-2	2-8	0.5-2	2-8

Pseudomonas aeruginosa ATCC® 27853

	MIC in		MBC in	
Drug	CAMHB[b]	CAMHB/HS[c]	CAMHB[b]	CAMHB/HS[c]
Piperacillin	4-16	2-8	8-32	4-16
Ticarcillin	32-128	16-64	32-128	16-64
Imipenem	1-4	2-8	4-16	8-32
Gentamicin	2-8	2-8	4.0-16	4-16
Tobramycin	0.5-2	0.5-2	1-4	1-4
Amikacin	2-8	2-8	4-16	4-16

[a]Although these quality control ranges are derived from a published study, it should be noted that this study was performed before NCCLS document M23–*Development of* In Vitro *Susceptibility Testing Criteria and Quality Control Parameters* defined the parameters for such studies.

[b]CAMHB = cation-adjusted Mueller-Hinton broth.

[c]CAMHB/HS = cation-adjusted Mueller-Hinton broth mixed with pooled human serum in a 1:1 ratio.

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Summary of Comments and Subcommittee Responses

M26-T: *Methods for Determining Bactericidal Activity of Antimicrobial Agents; Tentative Guideline*

Section 1.3.2.5

1. Is the loss of organism on the pipette tip negligible or should the tip be flushed several times with inoculum to saturate potential binding sites?

- **It is the opinion of the subcommittee that flushing the calibrated micropipette with inoculum is not necessary.**

Section 1.3.2.6

2. Last paragraph, two disparate ideas in a single paragraph.

- **The paragraph has been revised.**

Section 1.4

3. Paragraph 6 – "Kill-kinetic" is used without definition; should it be explicitly stated what kill-kinetic methodology is?

- **This term has been defined. Also see Section 2.3.**

Section 2.1

4. This section states that antimicrobial solutions can be kept in 1-mL aliquots at –70 ºC for periods up to six months. Real time stability data must be available to support the claim for each antimicrobial solution. The container closure system should also be specified, i.e., amber glass, clear borosilicate glass, Teflon® stopper.

- **Most laboratories successfully store antimicrobial solutions in screw-capped plastic tubes at -70 ºC.**

Section 2.2.2

5. Last paragraph — Given the emphasis on the use of the ultrafiltrate, this paragraph should be expanded to better explain what an ultrafiltrate is, what it contains (e.g., free drug), its safety (e.g., if patient is HBV and/or HIV positive), and potential suppliers of filtration devices.

- **Commercial sources change, and a listing of commercial suppliers is outside the purview of this document. Details of filter devices and sources are described in the following reference: Craig W, Gudmundsson S. Postantibiotic effect. *Antibiotics in Laboratory Medicine.* 4th ed. Williams and Wilkins Co.; 1996:2.**

Section 2.3.2

6. It is not clear how a larger volume presents a greater challenge. There is an increased absolute number of organisms, but aren't the concentrations of organisms and antimicrobic the critical factors?

- **Although antimicrobial concentration is paramount, the absolute number of microorganisms does influence the likelihood of a resistant mutant being present.**

Section 2.5.1.1 (2)

7. Should a fresh tip be used for each tube or should inoculation be done from lowest to highest concentration, i.e., is antibiotic carryover on the inoculating pipette a problem?

- **Antibiotic carryover is negligible if serial pipetting proceeds from the lowest to highest antimicrobial concentration.**

Section 3.2

8. How do you determine two standard deviations?

- **This information can be found in the any standard statistical reference text.**

Section 3.3.1 (1) - (4) and Section 3.3.2 (1) - (4)

9. In these sections, you repeat what has already been well described in Section 2.5.

- **Repetition in the form of step by step outline was done for clarity and emphasis.**

Section 3.3.1 (5) and Section 3.3.2 (5)

10. There is no reference to incubating the broth.

- **This information has been added to this version.**

Section 3.3.2

11. Lines 6 and 7 appear reversed, i.e., plate is incubated before lethal end point can be determined.

- **See response to Comment 10; this has been revised.**

General Committee Responses

12. Page 31 — It states: the addition of 2% NaCl to MHB is not recommended when MBC are to be determined. Does this modify previous recommendations about the performance of MIC, where 2% NaCl was a must?

- **No. The addition of 2% NaCl is required for an accurate MIC.**

13. Many viridans streptococci are capable of growing in broth with chain-lengths exceeding 100 cells. These chains are not easily disrupted; placing the culture (in a glass container) in an ultrasonic waterbath has little effect on chain-length. If the inoculum broth contains organisms with excessive chain-length, then the count of the initial inoculum in terms of colony-forming units/mL is a gross underestimate of the number of viable organisms/mL. I believe that this is a major technical factor in the irreproducibility of time-kill and associate methodology, particularly for viridans streptococci. The time period for which the organism is grown in broth prior to standardization of the inoculum using a McFarland standard will affect the initial chain-length obtained. (Factors such as: the length of the lag-phase resulting in a change of media formulation from agar-plate to broth; the degree of metabolic shutdown relating to age of the culture on the plate on which the broth was inoculated; and the time taken to reach opacity all affect the chain-length of organisms in broth.) Sonication of the sample used to assess the initial inoculum in such tests does go some way to addressing this technical factor, and if properly controlled should result in more reproducible methods.

- **The relevance of this comment has been noted.**

Related NCCLS Publications*

M2-A6 **Performance Standards for Antimicrobial Disk Susceptibility Test Sixth Edition; Approved Standard (1997)**. This document provides current recommended techniques for disk susceptibility testing, new frequency criteria for quality control testing, and updated tables for interpretive zone diameters.

M7-A4 **Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically Fourth Edition; Approved Standard (1997)**. This document provides reference methods for the determination of minimal inhibitory concentrations (MIC) of aerobic bacteria by broth macrodilution, broth microdilution, and agar dilution.

M11-A4 **Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard Fourth Edition (1997)**. This document provides methods for susceptibility testing of anaerobic bacteria; description of reference agar dilution method, alternative agar methods (Wadsworth and limited dilutions), broth microdilution, and broth (macro) dilution procedures, and quality control criteria for each procedure.

M21-A **Methodology for the Serum Bactericidal Test; Approved Guideline (1999).** This document provides a direct method of antimicrobial susceptibility testing using a patient's serum to measure the activity of the serum against the bacterial pathogen isolated from the patient.

M29-A **Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue; Approved Guideline (1997).** This document provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in any laboratory setting; specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials; and recommendations for the management of blood-borne exposure.

NOTES

* Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.

NOTES

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