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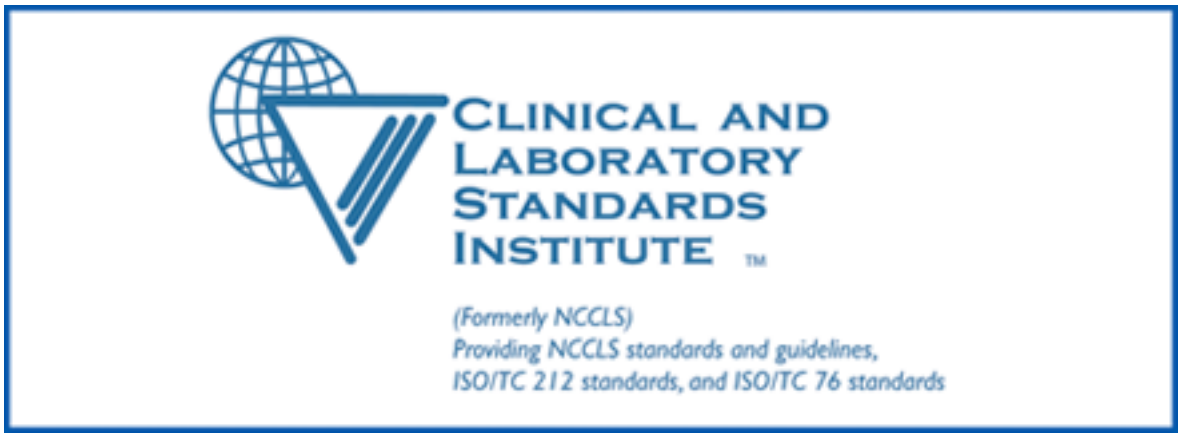
Methodology for the Serum Bactericidal Test; Approved Guideline



This document provides a direct method of antimicrobial susceptibility testing using serum obtained from the patient to measure the activity of the serum against the bacterial pathogen isolated from the patient.

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Methodology for the Serum Bactericidal Test; Approved Guideline

Abstract

Bactericidal testing methods occasionally are used because of the awareness of the need for lethal antimicrobial activity to treat certain deep-seated infections, e.g., bacterial endocarditis. There also is an increased awareness of the need to consider the pharmacodynamic and pharmacokinetic principles of antimicrobial therapy. This often can be achieved by assessing both results of standardized susceptibility testing and of antimicrobial assays. The serum bactericidal test represents an alternative *in vitro* test that incorporates pharmacodynamic and pharmacokinetic principles. The test takes into consideration the susceptibility of the pathogen; measures the combined effect of absorption and elimination of the antibiotic; the binding of the drug to serum proteins; and the effect of the parent compound as well as any metabolites against the infecting organism. This assay also measures the effect of drug interactions. This includes both the synergistic or antagonistic effects of antibiotic combinations and the interactions of other drugs with antibiotics. Finally, the serum bactericidal test, by measuring the magnitude of antibiotic concentration relative to the MBC, allows a prediction of the aggregate time of bactericidal activity. Peak serum bactericidal titers of > 8, for example, assure measurable bactericidal activity in the serum for at least three half-lives of the drug being tested. Despite all of these potential advantages, the serum bactericidal test rarely is needed. Although the test provides another predictive estimate of bacterial eradication, clinical cure depends largely upon host factors and other factors, e.g., postantibiotic effect and the growth-inhibitory effects of sub-MIC concentrations of antibiotics. When needed, however, established, reproducible methods should be available to the clinical, reference, and/or research laboratory.

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Volume 19 Number 17

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Foreword

All of the susceptibility test methods commonly performed by clinical microbiology laboratories (i.e., disk diffusion, broth dilution, and agar dilution) measure the inhibitory activity (MIC) of an antimicrobial agent.[1-3] 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]

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] and for osteomyelitis[7], as well as infections in immunocompromised patients.[8]

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.

The clinical relevance of the serum bactericidal test remains controversial, and there are relatively few clinical situations in which the test is indicated. However, the serum bactericidal test is being used in volunteers as a tool in the evaluation of new antimicrobial agents. Whatever the reason for performing the serum bactericidal test, this document describes the details of the test and, in particular, describes the methodologic variations. This information has been obtained largely from published data. Use of these techniques should result in uniform methodology that is practical enough to be used in the clinical microbiology laboratory or in the research setting. The techniques are intended primarily for testing aerobic bacteria that grow well after overnight incubation in Mueller-Hinton broth.

In depth discussion of the factors influencing results of bactericidal tests and techniques for the conduct of MBCs and timed-kill studies may be found in the most current edition of NCCLS document M26–*Methods for Determining Bactericidal Activity of Antimicrobial Agents.*[9]

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-

Foreword (Continued)

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

Schlichter test, serum bactericidal test, serum bactericidal titer, serum dilution test

Methodology for the Serum Bactericidal Test; Approved Guideline

1 Introduction

1.1 Principle

Bacterial eradication by antimicrobial therapy is determined both by the pharmacodynamic and pharmacokinetic properties of the antimicrobial agent(s) used.[10]

The pharmacodynamic properties of an antimicrobial agent are defined as its activity (inhibitory and/or lethal) against microbial pathogens. When this activity is evaluated over a time-concentration continuum, the lethal effect can be described either as concentration-dependent or time-dependent.

Concentration-dependent killing kinetics, as seen with aminoglycosides and fluoroquinolones, are those in which the rate of killing increases with increasing drug concen-trations up to a point of maximum effect. In contrast, time-dependent killing kinetics, as seen with β-lactam agents and vancomycin, are those in which the rate of killing is relatively slow and continues only as long as the concentrations are in excess of the MIC.

Because both the magnitude and duration of the activity of the antimicrobial agent can be important in determining its bactericidal effect, the pharmacokinetic properties of the agent as well as the dosing parameters become important factors in determining the likelihood of bacterial eradication.

The usual *in vitro* susceptibility test methods such as the MIC-MBC determination or broth time-kill kinetic methodology do not consider the pharmacokinetic properties of the agent, but instead rely upon known pharmacokinetic properties with the usual dosing regimens. Often, an antimicrobial assay can provide the necessary pharmacokinetic data which can be combined with the susceptibility test result to allow one to predict bacterial eradication. For certain infections (such as endocarditis) requiring bactericidal activity, the serum bactericidal test is a method that considers both the pharmacodynamic and the pharma-cokinetic properties of an antimicrobial agent and can provide another predictive estimate of bacterial eradication.

The serum bactericidal test can be used to predict the margin of bactericidal activity relative to the MBC at the beginning and end of the dosing interval. It also can be used to measure duration of killing by serially determining the level of bactericidal activity and the area-under-the-bactericidal-titer-curve.[11] Lastly it can effectively assess the rapidity of killing in serum by measuring the killing effect over time.[12]

In a clinical setting, the peak and trough serum bactericidal titers are most easily determined and usually provide sufficient clinical information. In a research setting, the area-under-the-bactericidal-titer-curve and the serum bactericidal rate may be determined in addition to the serum bactericidal titer in order to provide a more comprehensive evaluation of the pharmacodynamics and pharmacokinetics of a new antimicrobial agent.

1.2 Clinical Relevance

It must be understood that, like most susceptibility tests, there is very little published information documenting the clinical relevance of the serum bactericidal tests.

The serum bactericidal test most often has been used to evaluate the therapeutic effec-tiveness of antimicrobial agents in bacterial endocarditis.[13,14] In a comprehensive review of the literature covering the years 1948 to 1980, Coleman et al. were unable to find evidence that either the serum inhibitory or bactericidal titers had any prognostic value in the treatment of patients with endocarditis.[15]

However, evaluation of the serum bactericidal test in bacterial endocarditis is complicated by the fact that such patients receive four to six weeks of parenteral antimicrobial therapy and, for the most part, are cured by this regimen regardless of their serum bactericidal titers. For example, a multicenter collaborative study of infective endocarditis[16] required seven years to acquire enough patients in the study to have statistically significant results, yet had only nine clinical failures. This same study found that

peak bactericidal titers of 64 or greater with the microdilution method were associated with 100% bacteriologic cure. The often-recommended peak serum bactericidal titer of 8 was not associated with predictive accuracy and had no statistically significant association with clinical cure. Finally, the results of the serum bactericidal test in this study were unable to accurately predict the failure of bacterial eradication.

The serum bactericidal test also has been used to monitor intravenous therapy followed by oral antimicrobial therapy for infective endocarditis, and the clinical outcome of these patients was excellent.[17] The serum bactericidal test (both the serum bactericidal titers[18] and the serum bactericidal rate[19]) have proven useful in animal models and in human therapy for assessing combination therapy of bacterial endocarditis.

The serum bactericidal test also has been used to monitor antimicrobial therapy in cancer patients. Klastersky et al.[20] measured the peak and trough levels of bacteriostatic and bactericidal activity of the serum and urine of 317 patients with cancer and a bacterio-logically proven infection. When the serum bactericidal test had a peak titer of > 8, the infection was cured in 80% of cases.

Similarly, Sculier and Klastersky[8] analyzed the clinical significance of the serum bactericidal activity in cancer patients who presented with bacteremia caused by gram-negative bacilli. These investigators noted that the clinical response to antibiotic therapy in these patients strongly correlated with the peak serum bactericidal activity. Ninety-eight percent of nongranulocytopenic patients with a serum bactericidal titer of 8 or more had a favorable clinical response.

In contrast, patients with severe neutropenia (granulocyte count below 100/mm^3) had an 87% rate of cure only with titers of 16 or more. The higher titers needed with neutro-penic patients may be related to the need for bactericidal activity over a longer time span. A 16-peak serum bactericidal titer guarantees some bactericidal activity in the serum for four half-lives of the drugs being tested. Drusano et al.[21] have found that four of five neutropenic patients with gram-negative bacteremia in whom empiric combination therapy failed had no bactericidal titers at trough levels.[21] This is in distinct contrast to the 21 patients who survived, all having measurable serum bactericidal titers at trough levels.

The serum bactericidal test appears to have utility in some cases for predicting bacterial eradication in patients with skeletal infections. Osteomyelitis and suppurative arthritis remain difficult to treat successfully despite the availability of effective antimicrobial agents. Dich et al.,[22] for example, found that 19% of children whose antibiotic regimen was changed from parenteral to oral during the first three weeks of treatment had relapses or their infection took on a chronic course.

This is in contrast to the cumulative experience of other investigators who have used the results of the serum bactericidal test to adjust the therapy of children receiving parenteral followed by oral antimicrobial therapy. Tetzlaff and colleagues[23] treated 30 children with acute hematogenous skeletal infections (19 osteo-myelitis, 3 osteoarthritis, and 8 suppurative arthritis) with a brief (1 to 13 days) period of parenteral antibiotic therapy followed by oral therapy to complete a usual 4-week course. Adjustments in dosage were made when necessary to insure a peak serum bactericidal titer of at least 8. Twenty-nine of thirty were successfully treated.

Kolyvas et al.[24] also used the serum bactericidal test to monitor parenteral/oral therapy in children, adjusting doses of antibiotics to achieve peak titers of 1:8. All of 10 children studied remained cured after follow-up of one year. Prober and Yeager[25] reported the results of 22 children treated with sequential therapy of intravenous followed by oral agents. Peak serum bactericidal titers of 8 were achieved in 21 of the 22 patients; trough titers of 2 in 20 of 22 patients. The authors had no recurrences in 21 of 22 patients.

Finally, Syrogiannopoulous and Nelson[26] reviewed ten years of experience with children who had acute suppurative osteoarthritis. There were 180 children who received large doses of oral antibiotics following clinical stabilization with intravenous antibiotics; the median duration of intravenous therapy was about one week. Serum bactericidal titers were used routinely to monitor absorption and determine an adequate dose. These investigators used a peak serum bactericidal titer of 1:8 when the

pathogen was a gram-negative bacillus, *S. aureus*, or *H. influenzae* and at least 1:32 when the etiologic agent was a streptococcus. If the titers were suboptimal, the dosage of the antibiotic was increased. The dosages of oral antibiotics used under these guidelines were two-to-three times those normally used.

Over this ten-year period, no patient with suppurative arthritis was readmitted with recurrence. Four patients with acute osteomyelitis were readmitted with recurrence, representing 3.8% of the 106 patients with bone infection. In two of these recurrences, noncompliance with the oral therapy (one case proven and one suspected) were felt to be the reason for antimicrobial failure.

In a retrospective report of 18 adults with osteomyelitis, Black et al.[27] assessed the plausibility of sequential parenteral-peroral antimicrobial therapy, adjusting the trough titer on peroral therapy to >1:8. There were 16 out of 18 patients who were cured.

In another study with mostly adults, Weinstein et al.[7] prospectively assessed the value of serum bactericidal titers to predict outcome of patients with acute and chronic osteomyelitis. These investigators analyzed 51 patients with osteomyelitis, 30 acute and 21 chronic, who were monitored at multiple medical centers with the same serum bactericidal test methodology.

In this series, trough serum bactericidal titers of 1:2 or greater predicted medical cure in 23 of 25 patients successfully treated for acute osteomyelitis. There were 13 successfully treated episodes out of 21 cases of chronic osteomyelitis. In these 13 patients, peak serum bactericidal titers of 1:16 or greater and the trough titers of 1:4 or greater were achieved and accurately predicted a cure. In contrast, peak titers of less than 1:16 and trough titers of less than 1:2 in the 8 patients in whom therapy failed accurately predicted this failure.

Other investigators reported that one should aim for trough values of 1:2 or greater, whenever possible.[28] However, if the patient response is favorable, dosage should not be altered.

1.3 Clinical Use

It must be recognized that the number of clinical situations in which a serum bactericidal test is needed is very limited. There are, however, theoretical situations in which the serum bactericidal test might be useful.

The serum bactericidal titer, for example, can be used to determine the magnitude of serum bactericidal activity in relation to the MBC. This parameter can be important for antimicrobial agents that exhibit concentration-dependent killing kinetics as shown by Moore and colleagues[29] who have reviewed the clinical course of 236 patients with gram-negative bacillary infections that were treated with aminoglycosides. The clinical response to aminoglycosides in these patients was 80% (188 patients). Elevated maximal and mean peak aminoglycoside concentration/MIC ratios were strongly associated with clinical response ($P < 0.0001$ and $P < 0.00001$, respectively). By logistic regression the peak concentration/MIC ratios were associated significantly with clinical response after adjustment for underlying severity of disease and other factors correlated with response. The serum bactericidal test, conceptually, measures no more than the serum level of the antimicrobial agent divided by the MIC or MBC of the infecting organism.[30]

NOTE: This statement is generally true providing there is no chemical, ionic binding, or pharmacologic interference with drug activity.

There are some conditions where drug concentration does not reflect drug activity. Although for aminoglycosides the availability of assay methods for determining the peak levels precludes the need for the serum bactericidal test, the serum inhibitory activity for peak levels of aminoglycosides, in theory, should be useful in predicting clinical response in patients with gram-negative bacillary infections who are treated with aminoglycosides. There are no currently available assay methods similar to those available for aminoglycosides for fluoroquinolones. Fluoroquinolones are also concentration-dependent agents. Determination of serum bactericidal titers therefore may be useful in intravenous therapy with ciprofloxacin so as to avoid clinical failure as has been reported for seven of ten patients with nosocomial pneumonia caused by *P. aeruginosa* who were treated with intravenous ciprofloxacin.[31]

Moore and colleagues also noted that high plasma levels of aminoglycosides are associated

with decreased mortality in patients with gram-negative bacillary pneumonia.[32]

For antimicrobial agents that exhibit time-dependent killing kinetics, a trough serum bactericidal titer would, in theory, be useful to demonstrate that the serum concentration-time profile has free drug levels always remaining above the MIC (e.g., four-to-eight times the MIC) for the duration of time prior to the next dose. The clinical relevance of such an approach can be seen in the study of Warren et al.[33] who found that the efficacy of cefoperazone correlated best with the trough concentration of free drug being above the MIC of the infecting isolate.

The serum bactericidal test can also be used to measure not only the magnitude of serum bactericidal activity but also its duration. This is done by plotting serum bactericidal titers measured over time and calculating the area under the curves. The largest area under the bactericidal-titer curve will be found for the antimicrobial agent(s) with the best combination of three factors: antibacterial activity, achievable free drug concentrations, and half-life.[11]

Barriere et al.[34] serially determined the serum bactericidal activity of three new cephalosporins and found prolonged bactericidal activity against *E. coli* for cefotaxime despite the half-life of this agent being only 1 hour. The authors postulated that the unexpected prolonged bactericidal activity was likely due to the presence of the active metabolite, desacetylcefotaxime, and suggested that a dosage of 1 g of cefotaxime every 8 to 12 hours should be sufficient for infections caused by susceptible gram-negative bacilli. The efficacy of this dosing schedule subsequently has been confirmed by the clinical study by Goodpasteure et al.[35] The area under the bactericidal-titer curve provides another dimension to the evaluation of investigational antibiotics.

The serum bactericidal titer, like the MBC, determines bactericidal activity after 24 hours of incubation and separates the bacteria into two populations, a segregation that might not have biological relevance. The serum bactericidal rate, however, evaluates the rapidity of bacterial killing and has been shown in an experimental animal model to correlate better with the rapidity of sterilization of vegetations and to reflect the concentration dependency of the synergy of nafcillin plus an aminoglycoside against *S. aureus*.[19,36,37]

In human volunteers, Van der Auwera et al.[38] compared the serum bactericidal rates for imipenem and vancomycin against methicillin-susceptible and methicillin-resistant *S. aureus*. These investigators found that against methicillin-susceptible *S. aureus* isolates, imipenem and vancomycin provided an equivalent rate of killing. Against methicillin-resistant isolates, imipenem was almost equivalent to vancomycin. The relevance of these findings is suggested by Fan et al.[39] who studied the clinical efficacy of imipenem against methicillin-susceptible and methicillin-resistant *S. aureus*. Eleven of 12 methicillin-susceptible and 10 of 11 methicillin-resistant *S. aureus* infections were clinically cured. This method for evaluating the serum bactericidal rate is worthy of further investigation as a tool for the evaluation of new antimicrobial agents.

1.4 Interpretation

The relation of the test results of the serum bactericidal test to the clinical outcome is not such that cure or failure can be predicted easily.[40] Bactericidal activity is only one factor in the treatment of infected patients. It is important to understand the difference between bacterial eradication and clinical cure. Obviously, the ultimate clinical cure cannot be predicted accurately solely by an *in vitro* test of antimicrobial activity. Clinical cure depends largely upon host factors and other factors, e.g., postantibiotic effect and the growth-inhibitory effects of sub-MIC concentrations of antibiotics may also impact bacteriologic response of patients.[41]

Most immunocompetent hosts with bacterial infections, moreover, do not require antimicrobial therapeutic programs in which there is bactericidal activity. Strict adherence to the achievement of an arbitrary serum bactericidal activity in the absence of supporting data may create an increased risk for the patient. For example, drug toxicity might ensue due to an unnecessary increase in dosage in a clinically stable patient with a low titer.

Conversely, an effective dose may be lowered inappropriately. It is also important to realize

that the final result of the serum bactericidal test can be greatly influenced by methodology. Many factors must be carefully controlled if reproducible results (intralaboratory and interlaboratory) are to be achieved. Because of the specialized nature, the variations of methodology, the need for timed specimens, the complexity of technical factors, and the potential difficulty for interpretation, it is recommended that the serum bactericidal test be done in consultation with the director of the clinical microbiology laboratory (i.e., this test should be performed in reference or research laboratories or those laboratories with highly-trained medical technologists).

With such assistance, the clinician can more easily determine if the serum bactericidal test is the appropriate test for assessing bactericidal activity for his patient, determine which variation is most appropriate, assure that the serum specimens are collected properly, and properly interpret the results.

2 Collection of Patient's Serum

2.1 Peak and Trough Levels

Generally, both a peak and trough level are obtained with the serum bactericidal test. The optimal timing of samples and most appropriate storage conditions for collecting and obtaining peak levels varies by drug and by route of administration; therefore, the product labeling should be consulted to select the optimal sampling times.

This timing attempts to ensure that the peak level is obtained approximately 30 to 60 minutes after the antimicrobial agent is absorbed and distributed. The trough level is that level obtained immediately before the next dose.

These definitions are useful for single antimicrobial agent therapy and are based on the usual pharmacokinetic properties of antimicrobial agents. If more than one antibiotic is being used, the peak sample should be obtained 1 hour after administration of the second antibiotic. If one of the agents is administered infrequently (e.g., every 12 hours) and the other is administered more frequently (e.g., every 4 hours), peak levels should be obtained 1 hour after completion of the infusion of the second antimicrobial agent at a time when the administration of both antimicrobial agents coincides. Though levels may be obtained based on the timing of the less frequently administered agent, the assumption is made that the agent administered every 4 hours will have a more constant level.

2.2 Sample Collection and Handling

The serum specimen must be collected aseptically. Any bacterial contamination from the skin may interfere with the results. Also, because of short half-lives of some antibiotics, the timing of the serum collection is critical, and the time the antimicrobial agent was given should be confirmed. This should include:

- The time that the previous dose was given.
- The time that the current dose was given or will be given.
- The duration of the infusion when obtaining peak levels for intravenously administered agents.

Antimicrobial agents may not be given at the exact times ordered on a busy nursing unit, and care must be taken to ensure that peak and trough specimens are obtained accurately. Some hospitals designate one person to collect all samples for any type of drug assay. Specimens should be clearly marked with the following:

- Patient's name and hospital number.
- Antimicrobial agent(s) and dose(s) being used.
- Time of antimicrobial dose (start and completion of infusion) and initials of medication nurse.
- Time and date of specimen collection and initials of collector.

One method to ensure compliance with proper collection is to require that the above material be completed on a requisition slip by the appropriate persons (medication nurse and blood collector) and initialed. If the requisition slip is not properly completed (with initials), the specimen is not processed. After the specimen has been collected, serum should be separated from red blood cells as soon as possible. Rapid

transportation to the laboratory is necessary; if any delay is expected, the specimen (blood or serum) should be transported on ice. (Serum must be frozen if the tests are to be delayed more than 2 hours; blood cells must be separated first because freezing hemolyzes the red blood cells.) Adhere to the pharmaceutical manufacturer's guidelines for stability of the antibiotic in frozen human serum prior to testing. If serum is submitted frozen to a reference laboratory for SBT testing, send in a sterile plastic vial. Do not use glass.

2.3 Patient s Isolate

The patient's isolate must be saved to serve as the inoculum. The isolate may be kept frozen at −70ºC in tryptic soy broth or in a glycerol suspension of a subculture on a tryptic soy agar slant. 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.

Since variations in density of inoculum may alter the endpoints by one or more dilutions, the correct preparation of a standardized inoculum is critical. The rate of loss of viability of bacterial cells following exposure to most antibiotics is a direct function of bacterial growth prior to the addition of the agent with especially marked effect shown for cell-wall-active agents. 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 susceptible to the agent and, therefore, cause diminished killing rates. See references and text in the most current edition of NCCLS document M26—*Methods for Determining Bactericidal Activity of Antimicrobial Agents.*

In addition, the use of a lag phase cultures, i.e., cultures which have recently undergone a change in test condition (e.g., 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). A higher inoculum size, even in log phase cultures, will approach the stationary growth phase and such organisms may be killed more slowly.

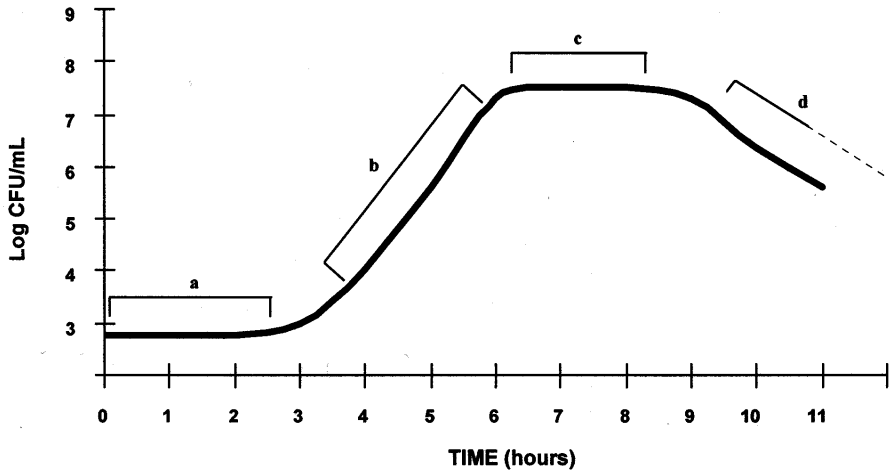

Figure 1. Bacterial Growth Curve. Growth curve of typical bacterial culture as measured by viable count (a: Lag phase; b: logarithmic phase; c: stationary phase; d: death phase).

3 Serum Dilution Procedure

The method for determining serum bactericidal titers is a variation of the broth dilution test, and many of the methods used to control the variables in the broth dilution test are applicable to the serum dilution procedure. Hence, it is advisable to refer to extensive discussions, particularly of biological and technical factors influencing test interpretation in NCCLS document M26—*Methods for Determining Bactericidal Activity of Antimicrobial Agents.*

3.1 Broth Medium

3.1.1 Human Serum as Diluent

In the serum bactericidal test, a broth is used to dilute the patient's serum. Many different broths have been used as diluents, including trypticase soy, brain heart infusion, Columbia, dextrose phosphate, and (most commonly) Mueller-Hinton.[41] The use of salt-containing diluents must be avoided when testing staphylococci and β-lactam agents. Human serum in a 1:1 ratio (CAMHB/HS) has also been recommended as the diluent.[42] The main rationale for human serum as a diluent is to assess the role of protein-binding in the results.[30,42,43] Although the *in vitro* effects of protein-binding are well known,[44,45] there has been debate as to the clinical relevance of this phenomenon. There is information available, however, as to the importance of protein-binding in determining clinical cure.

Chambers et al.[46] found that cefonicid, a highly protein-bound cephalosporin, failed to clear the blood of bacteria or to produce a resolution of clinical findings by day five of therapy in three of four patients with staphylococcal endocarditis. This was despite peak concentrations that were assayed in two patients and found to be 20 to 40 times the MIC for the infecting isolate. However, when protein was added to the medium, MICs for the isolates rose four-to-eightfold and bactericidal titers were < 1:8 at peak for all three patients.

In another study evaluating a highly protein-bound antimicrobial agent (teicoplanin), Calain and colleagues[47] described six failures despite measured trough concentrations of total teicoplanin which exceeded the MIC for the infecting pathogen by 5 to 35 times in four of these patients. Because teicoplanin is very highly protein-bound, free antimicrobial agent was noted to be less than the MIC even at peak concentrations. Bolivar et al.[48] evaluated cefoperazone (12 g/day) as single agent therapy in cancer patients. These investigators were able to achieve success in 80% (44 of 55) of cases where the MIC of cefoperazone was ≤ 25 μg/mL for the infecting organism.

Based on these clinical observations, Peterson et al.[49] have used pharmacokinetic concepts to predict what a 12-g/day dose of cefoperazone would produce in terms of extravascular levels of cefoperazone. They predicted that such a dose would result in extravascular levels of free cefoperazone in the range of 15 to 25 μg/mL. (Total mean levels in serum would be expected to range from 75 to 250 μg/mL.) This correlates very nicely with the clinical observations of Bolivar. Clearly, the clinical importance of protein-binding can be demonstrated for both gram-positive and gram-negative organisms.

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 the patient's serum.[50] This separates the free antimicrobial agent 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 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-micron filter.

For antimicrobial agents that are known to have relatively little protein binding (<90%, e.g., gentamicin and vancomycin), Mueller-Hinton broth can be used as the diluent. If there is appreciable protein-binding (>90%, e.g., ceftriaxone and nafcillin), then either an ultrafiltrate of the patient's serum or the use of pooled human serum as the diluent is recommended. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of most antimicrobial agents determined with and without human serum against strains of bacteria commonly used as quality controls in susceptibility testing can be useful for quality control measures (see Table 3).[51]

3.1.2 Broth Monitoring

The performance and chemical characteristics of both Mueller-Hinton broth and human serum must be routinely monitored.

- The pH of each batch of Mueller-Hinton broth should be checked with a pH meter

when the medium is prepared; pH should be between 7.2 and 7.4.

- The MIC characteristics of broth should be evaluated periodically with a standard set of quality control microorganisms and with an antibiotic from each major class. The frequency of such testing will depend on the overall quality control program and the volume of serum bactericidal tests done.

MICs for aminoglycosides and fluoroquinolones tested in Mueller-Hinton (MH) broth may be lower than MICs obtained on MH agar (particularly when *Pseudomonas aeruginosa* is tested) unless the MH broth is adjusted to contain the recommended concentrations of Ca^{+2} and $Mg.^{+2}$

Pooled human serum can be obtained from commercial sources or from volunteers. It must be quality controlled as rigorously as any other reagent 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-1. (The pooled human serum may be heated to 56 °C for 1 hour upon receipt to inactivate HIV-1 and complement.) Next, the serum should be adjusted with 0.1 N NaOH or 0.1 N HCl to pH 7.2 to 7.4. After adjustment of pH, the serum should be clarified by prefiltering with a 0.80-micron filter. Finally, the serum should be filtered using a 0.22-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 especially when β-lactamases are to be tested. There are several commercially available rapid β-lactamase tests. Any of these, except those utilizing PADAC[52] can be used by dropping some of the sera on the test strip and observing for a color change over 30 minutes. The serum should be screened for nonspecific antimicrobial activity by putting 20 µL of the pooled human serum on blank paper disks, placing the disks onto nutrient agar that has been seeded with a spore suspension (5×10^8) of *Bacillus subtilis* ATCC® 6633, and looking for zones of inhibition after incubation at 35 °C for 24 hours.

A final quality control measure is to periodically determine MICs and MBCs of various antimicrobial agents for control strains of bacteria in media supplemented with the serum. Use one antibiotic from each major class (penicillins, cephalosporins, and aminoglycosides) against an appropriate ATCC strain (see Section 7.3). MICs and MBCs of many antimicrobial agents have been determined with and without human serum against strains of bacteria commonly used as quality controls in susceptibility testing.[51] The pooled human serum should be heated to 56 °C for 1 hour just before use in order to inactivate HIV-1 and any complement. The serum may be stored at -20 °C or less until needed.

3.2 Dilution Methods

Inhibitory activity is determined by dilution methods similar to those used to determine an MIC. Dilutions having no visible growth can then be sampled with a calibrated device to determine the concentration at which 99.9% of the final inoculum is killed.

Serum bactericidal titers can be determined with:

- A macrodilution method (1 to 2 mL in each test tube).

- A microdilution method (100 µL in each well).

It can be difficult to determine 99.9% killing of the final inoculum with the microdilution method. However, the microdilution method has been found to be more reproducible than the macrodilution method for bactericidal testing.[51,53-60] Clinical studies suggest, moreover, that the microdilution method is useful.[7,8,10] It also is better suited for research purposes when testing serum from volunteers receiving antibiotics against large numbers of different isolates. Therefore, the microdilution method is recommended for the serum bactericidal test.

3.2.1 Macrodilution Method

The macrodilution method, if done, 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 procedure is similar to that for MIC determinations.

(1) Add 1.0 mL of the patient's serum to each of the first two tubes. Alternatively, use 1.0 mL of an ultrafiltrate of the patient's serum.

(2) Add 1.0 mL of heat-inactivated pooled human serum to tubes 2 through 10. Alternatively, use cation-adjusted Mueller-Hinton broth (CAMHB).

(3) Make serial two-fold 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). Make the dilutions 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.

(4) After the dilutions have been completed, add 1.0 mL of CAMHB, or other broth (if necessary) to each tube in order to yield a final volume of 2.0 mL in each tube, ending with a 1:1 ratio of pooled human serum to broth.

(5) Use the tenth tube as a control for growth of the organism.

3.2.2 Microdilution Method

The preferred 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 bacteria to the sides of the wells has not been found to be a problem as has been found with the use of plastic test tubes for the macrodilution method. The micro-dilution method is simple and more efficient, and requires less broth. The following proce-dure is similar to that for MIC determinations.

(1) Add 0.05 mL of pooled human serum or CAMHB to each well of columns 2 through 10 in each row.

(2) Add 0.05 mL of the patient's serum or serum ultrafiltrate to each of the first two wells.

(3) Make serial two-fold dilutions of the serum from column 2 through column 9 with a semiautomatic microdiluting device employing 0.05-mL microdiluters.

(4) Use the tenth well as a positive growth control.

3.3 Preparing Inoculum

The patient's isolate is used to prepare the inoculum. Variations in density of inoculum may alter the endpoints by one or more dilutions, and therefore, the preparation of a standardized inoculum is critical.

(1) To address the possibility of heterogeneously distributed resistance among colonies, prepare the standardized final inoculum by using 5 to 10 colonies of a single type from a 16- to 24-hour agar plate containing nonselective culture medium; inoculate them into a tube containing 5.0 mL of prewarmed (35 °C) broth (i.e., CAMHB or trypticase soy broth).

(2) Incubate this bacterial suspension at 35 °C until it is visibly turbid (up to six hours for staphylococci; gram-negative rods may require < six hours). Log-phase inocula should be prepared in a shaker-incubator and flask or beaker, whenever possible, 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.

(4) Dilute the adjusted culture in broth (macrodilution method) or 0.9% buffered saline (microdilution method) so that after inoculation of drug-containing broths, each tube or well contains 5×10^5 CFU/mL.

The number of CFU/mL in the broth medium just before incubation is known as the final inoculum. The inoculum size of the final inoculum must be determined by serial dilution in saline and subculture to solid media for use the next day to interpret killing endpoints. 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 tubes is 2.0 mL, and the inoculum is 0.1 mL, the adjusted culture (1.5×10^8 CFU/mL)

must be diluted 1:20 with broth to yield 7.5 x 10^6 CFU/mL. When 0.1 mL of this suspension is inoculated into 2.0 mL of the broth, the final inoculum of bacteria will be approximately 4 x 10^5 CFU/mL.

Count the viable colonies in the inoculum to verify the final inoculum size. (This can easily be done by using the growth control test tube or by using a second growth control well in the microdilution plate.) This is accomplished by using a micropipettor to drop a 10^{-2}, 10^{-3}, and 10^{-4} saline dilution of the inoculum onto the surfaces of agar plates and then spreading this over the surface. (The micropipettor volume should be ≤ 0.1 mL.) After overnight incubation, the plate showing 20 to 200 colonies is used to calculate the initial inoculum size. For example, if there are 40 colonies on the plate inoculated with the 1:1000 dilution, then the final inoculum contained 4 x 10^5 CFU/mL.

3.4 Inoculating Broth

3.4.1 Macrodilution Method

Prepare the dilutions of the patient's serum before adjusting the actively growing inoculum. Within 15 minutes after the inoculum has been standardized, add 0.1 mL of the adjusted inoculum to each tube in the dilution series with a micropipettor to release the inocula beneath the surface of antimicrobial-containing solution. Mix by flushing two or three times without creating air bubbles or splashing the sides of the tubes. Do not vortex or agitate the tubes.

3.4.2 Microdilution Method

As in the macrodilution method, make the dilutions before adjusting the actively growing inoculum. Then, dilute the inoculum and use to inoculate the solution within 15 minutes after the inoculum is standardized. If the volume of the inoculum exceeds 10% of the well volume, the diluting effect of the inoculum on the antimicrobial agent must be taken into account. If a 0.05-mL pipette dropper is used to add inoculum to the wells, the resulting dilution is 1:2 which results in a final range of dilutions of the patient's serum of 1:2 to 1:512.

An alternative way to inoculate the wells is to first add 0.05 mL of broth to each well after the dilutions of the serum have been made and then add 0.0015 mL of inoculum with a multipoint inoculator and a seed tray. In this case, the number of organisms in the seed tray should be determined and must be at least 5 x 10^7 CFU/mL. To prevent evaporation during incubation, seal each tray in a plastic bag, with plastic tape or with a tight-fitting plastic cover before incubation.

3.5 Incubation

For all test methods, incubate the tubes or trays at 35 ºC in air or CO_2, if required for growth of the patient's isolate. Macrodilution tubes should be incubated for 20 hours, vortexed, reincubated, and then vortexed again before sampling at 24 hours. Microdilution trays should be incubated for 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, do not stack microdilution trays more than four high.

3.6 Determining Endpoints

The commonly used definition of serum bactericidal activity is 99.9% killing (□ 3 $\log_{10}$ drop in CFU/mL) of the original inoculum. The final inoculum size must be known. The volume subcultured must be based on this final inoculum size and must be done quantitatively. Because of inaccuracy due to random variation in the number of cells 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 the antimicrobial agent is carried over in a concentration high enough to continue inhibition of viable cells. 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.[50-57]

In these techniques, the final inoculum size can be determined by a method such as the surface-drop count[58] or by a quantitative subculture using a calibrated pipettor.[50] Rejection values are determined by a chart that considers the final inoculum size, single or double sampling, pipetting error, and the Poisson distribution of sample responses (Tables 1 and 2 contain the rejection values, i.e., the threshold for assessing lethal activity, for single and duplicate 0.01-mL sampling with a 5% pipette error[50]). The calculated sensitivity and specificity for these rejection values are

provided for the sake of completeness. (See footnote (a) in Table 2.)

3.6.1 Macrodilution Endpoints

Determine macrodilution endpoints 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 of CFUs.

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

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

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

(7) Streak the broth subcultured on an agar plate. The number of colonies subsequently grown after 48 hours of incubation is used to determine the lethal endpoint based on the final inoculum and the rejection values. 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 (Table 2).

(8) The dilution which demonstrates 99.9% killing of the final inoculum is the bactericidal dilution titer.

3.6.2 Microdilution Endpoints

(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 of CFUs.

(4) The final inoculum can be added to the wells in a volume equal to half the final volume or can be added using a multipoint inoculator (0.0015 mL). Do not shake.

(5) A multipoint inoculator or a pipettor can be used to remove 10 µL; duplicate subcultures should be done.

(6) Spot the broth subcultured with the multipoint inoculator on an agar plate with the first *no growth* after 48 hours of incubation being considered the lethal endpoint. Alternatively, streak the broth subcultured on an agar plate and after 48 hours of incubation, count the colonies and use the rejection values in Tables 1 and 2. Colonies should not be counted when the 10 µL is spotted on an agar plate due to the tendency for the colonies to run together.

(7) The dilution which demonstrates 99.9% killing of the final inoculum is the bactericidal dilution titer.

3.7 Interpretation of Bactericidal Dilution Titers

Although interpretation of the bactericidal dilution titers is controversial based on the limited number of clinical evaluations, the following are "approximate" guidelines for the clinical laboratory that uses the microdilution methodology delineated herein. As a general guideline, SBTs of 16 to 32 were frequently associated with more favorable clinical outcomes in patients with endocarditis.[7,8,28] The peak SBT has been predictive of favorable outcome for some patients with skeletal infections although some investigators have indicated that trough SBTs of 2 or greater may be more predictive.

Inability to achieve a specific titer without toxic levels of the antimicrobial agent(s) may indicate the need for alternative agents. Lastly, the medical literature should be followed for other applications and validations.

4 Area Under the Bactericidal-Titer Curve

The area under the bactericidal-titer curve is determined by plotting bactericidal titers

measured over time and calculating the area under the curve. The resulting area under the bactericidal-titer curve serves as a unifying value to measure not only the magnitude of serum bactericidal activity but also its duration. The largest area under the bactericidal-titer curve is found for the antimicrobial agent with the best combination of three factors: antimicrobial activity, achievable free-drug concentration, and half-life. The individual serum bactericidal tests which are performed over time are done as described in Section 3. This technique is generally reserved for research as a means of comparing different antimicrobial agents or combinations of agents.

5 Serum Bactericidal Rate

The serum bactericidal rate evaluates the rapidity of bacterial killing over time. The serum bactericidal rate is performed by first obtaining peak and trough serum samples which are then processed to yield ultrafiltrate. The ultrafiltrate is filter-sterilized and then mixed with an equal volume of CAMHB to yield a 1:2 dilution. A total volume of 10 mL is used with a glass test tube. The inoculum is added in a small volume below the surface with a micropipettor in order to avoid splashing on the inside of the test tube above the meniscus. The final inoculum should be approximately 5 x 10^5 CFU/mL and confirmed by a colony count. Test tubes should not be agitated during incubation, but should be vortexed after each sampling.

One test tube is used as a growth control and consists of the same inoculum added to CAMHB. The final inoculum is confirmed at time 0; subsequent colony counts are done at 4 hours, between 6 and 12 hours, and at 24 hours. Sampling for colony counts is done by removing 0.5-mL samples of the broth at the specified times. The 0.5-mL sample is serially diluted in test tubes containing 4.5 mL of buffered sterile saline (0.9% NaCl) to produce tenfold dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}). A number of different colony count methods can be used to determine the CFU/mL from these dilutions. One-milliliter samples of the serial dilutions can be added to a tube of molten agar (48 ºC) for the preparation of a pour plate.

Alternatively, 0.1-mL samples of the serial dilutions can be pipetted onto prewarmed (35 ºC for 1 hour) agar plates and distributed evenly using sterile, bent glass rods. Yet another method uses 20 to 50 mL samples which are dropped on each of five spots onto prewarmed agar plates and allowed to absorb without streaking.

Whichever method is used, the minimal, accurately detectable number of CFU/mL must be determined by serial dilutions with a known inoculum. Potential drug carryover (Section 6) must be evaluated and, if a problem, steps taken to eliminate these effects. Colonies are counted after 24 to 48 hours of incubation. A magnifying lens can facilitate colony counts when plates are incubated for 24 hours. Prolonged incubation (48 hours) facilitates counting colonies as the colonies are larger. With certain organism/ antibiotic combinations, the resultant colonies may be changed (i.e., dwarf size and not readily detected). Prolonged incubation of plates before colonies are counted may be more accurate. The kill-kinetic determinations are shown graphically by plotting $\log_{10}$ CFU against time. A bactericidal effect can be seen by a ≥ 3 $\log_{10}$ CFU decrease (99.9% killing of the final inoculum) at the time specified.

During the performance of these studies, colony counts may show a final increase in number after an initial decrease. This can be due to the selection of resistant isolates (most often phenotypic resistance[61]), inactivation of the antimicrobial agent (most likely after 24 hours), or regrowth of susceptible bacteria which have escaped the antimicrobial activity by adhering to the wall of the test tube.[57,62,63] 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.

For comparisons of serum bactericidal rates, linear regression analysis can be done[12] with the serum bactericidal rate being defined as the slope of the regression line, and its units being the change in the $\log_{10}$ CFU per milliliter per hour of exposure to the agent. The more negative the slope, the faster the rate of bacterial killing. A positive slope (e.g., in controls) indicates growth. Alternatively, the area-under-the-killing-curve[64] can be determined. Unlike linear regression analysis, the latter method allows for nonlinear segments in the curves. The serum bactericidal rate has mainly been used as a research tool, but has potential usefulness in clinical situations.[19,36]

6 Antibiotic Carryover

The transfer of a quantitated volume of broth from the serum inhibitory titers or from a kill-kinetic determination in order to assess the number of survivors can be complicated by antibiotic carryover.[65] This problem occurs mainly when higher concentrations of antibiotic are present and is especially common when testing β-lactam agents and staphylococci. Antibiotic carryover can be detected by streaking the sample subcultured across the surface of a prewarmed agar plate, allowing 20 minutes for antimicrobial absorption into the agar, then cross-streaking the test organism 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.[60] This effect can be minimized by allowing inoculum to dry on the agar surface before spreading.

7 Quality Control

7.1 Purpose

The quality control program monitors:

- The precision and accuracy of the susceptibility test procedure, including the inoculum size and the growth of the microorganism.
- The performance of reagents.
- The individuals who perform the test and read the results.

This goal is best realized through the use of reference strains selected for their genetic stability and for their usefulness in the particular method being controlled.

7.2 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 occasional testing of the media used in bactericidal testing and for occasional testing of individuals who perform the test.

7.3 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. Some strains with useful endpoints, however, have been tested repeatedly and have proven to be stable. 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.

For suitable quality control ranges, see Table 3.

7.4 Batch or Lot Control

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

7.5 Other Control Procedures

7.5.1 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 endpoints.

7.5.2 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. Also, the growth control can be subcultured at the same time that the serum bactericidal test is performed in order to assess purity.

7.5.3 Reproducibility

If serum dilution titers are determined by the microdilution method, the reproducibility can easily be determined by performing the test in duplicate.

7.5.4 Paradoxical Effect

The paradoxical effect is defined as the occurrence of progressively increasing plate counts for at least three consecutive concentrations above the serum inhibitory titer. If this occurs, the concentrations exhibiting the paradoxical effect are usually ignored for *S. aureus* but are reported when dealing with those gram-negative bacteria which can be inducible/constitutive producers of Class C (Group 1) β-lactamase (e.g., *Enterobacter, P. aeruginosa*). Paradoxical effect can also be seen with quinolones once the maximum bactericidal concentration has been exceeded.

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)	5% Error (Pipette error plus full sampling error) for Determination of Final Inoculum[b] Rejection Value[c]	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 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 (a 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

M21-T: *Methodology for the Serum Bactericidal Test; Tentative Guideline*

General Comments

1. I would argue that a critical review of the clinical relevance of the serum bactericidal test to the clinical setting be a part of the document.

- **Serum bactericidal tests by different methods have been used for 50 years; however, critical assessments of clinical utility are few and not sufficient. A principal goal of an approved NCCLS serum bactericidal testing guideline is to foster the very studies that could become references in future documents.**

2. While I have many concerns about the use of this test in most clinical laboratories, I believe it is possible to obtain reproducible answers depending on the technology that is employed in performing the test.

- **Ensuring reproducibility of results with this testing methodology is the main objective of this serum bactericidal testing guideline.**

3. We avoid the use of serum additive because it introduces a major, uncontrolled variable to the test, and the direct effects of serum on antimicrobial agent performance due to protein binding during patient treatment can be estimated easily. Because of the poor reproducibility of the MBC test, it should always be run in duplicate. When the macrodilution method is used, the subculture should be repeated after 48 hours of incubation if the results at 24 hours show skips or are discrepant. Also, we do not recommend any agitation before sampling.

- **Using the microdilution methodology in this document should enable a reproducible result. An ultrafiltrate of the patient s serum is an acceptable alternative to dilution with pooled human serum as noted.**

4. Since this test has quite limited scientific evidence correlating a single or specific titer to therapeutic outcome, I would strongly encourage NCCLS in the next revision of this document to not publish specific interpretive criteria.

- **This comment is duly noted. The importance of keeping abreast of the medical literature on this topic has been emphasized in this version.**

5. 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. 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 lab phase resulting in a change of media formulation from agar-plate to broth, the degree of metabolic shutdown relating the age of the culture on the plate 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.**

Foreword

6. Last sentence - Should this read "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"?

- **The wording in the Foreword has been revised accordingly.**

Section 1.1

7. Is "integrates" the appropriate word, or would something like "incorporate" be more accurate? The word "integrate" is used repeatedly throughout the document in this same sense.

- **The indicated wording has been revised accordingly.**

8. The serum bactericidal test which can determine the magnitude of bacterial activity. . .The word "magnitude" does not read well and is repeated.

- **This comment has been noted and the text revised.**

Section 2.2

9. Add a sentence for those physicians submitting to referral laboratories. We suggest. . ."If submitted frozen to a referral laboratory for testing, submit in a sterile plastic vial. Do not use glass."

- **This comment has been noted and the text revised.**

Section 3.1

10. The sentence "The greatest effect. . ." is awkward; suggest rewording.

- **This comment has been noted and the text revised.**

11. Could a list of such agents affected by protein binding be provided (recognizing that new agents are always being released)?

- **This comment has been noted and the text revised.**

Section 3.1.2

12. With the emphasis on use of the ultrafiltrate, could these details be deleted or relegated to an appendix?

- **Details on the use of the ultrafiltrate should be in the text itself for laboratories that use pooled human serum.**

Section 3.3

13. It is not necessary to prepare the inoculum by using 20 to 30 colonies. We suggest that 5 to 10 colonies would work as well.

- **This comment has been noted and the text revised.**

14. It is suggested, when attempting to count viable colonies from the growth control well inoculum and using a micropipettor to drop dilutions, that this be done in duplicate and the numbers of colonies be counted and averaged to give a better representation of initial inoculum size.

- **This comment has been noted and the text revised.**

Section 3.5

15. Specify atmospheric conditions of incubation, i.e., ambient, CO_2, or other.

- **The atmospheric conditions have been added to the text.**

Section 3.6

16. The term "rejection values" is not clear. Define the term and how it should be used.

- **A working definition of this term has been incorporated into the text of Section 3.6.**

Section 6.0

17. There are no instructions for preparing a diluted sample.

- **Detailed instructions regarding diluted sample preparation have been added to Section 6.**

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 minimum 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.

M26-A **Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guideline (1999)**. This document provides procedures for determining the lethal activity of antimicrobial agents.

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.

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

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