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Highly Reproducible Bactericidal Activity Test Results by Using a Modified National Committee for Clinical Laboratory Standards Broth Macrodilution Technique

DONNA M. HACEK,† DANA C. DRESSEL,† AND LANCE R. PETERSON†,*

Department of Pathology, Clinical Microbiology Division,† and Department of Medicine, Infectious Disease Division,‡ Northwestern Memorial Hospital and Northwestern University Medical School, Chicago, Illinois 60611

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Bactericidal testing historically has exhibited variable reproducibility, even when prior standardized methods were employed. Several modifications to the National Committee for Clinical Laboratory Standards (NCCLS) broth macrodilution method are proposed to improve reproducibility. Recommended changes from the approved NCCLS guidelines (M21-A and M26-A) include omitting serum supplementation of Mueller-Hinton broth, incubating tubes at 35°C for 24 h with no agitation until they are sampled, running all tests in duplicate with six dilutions instead of nine, reincubating the test for an additional 24 h to resolve discrepant bactericidal activity test results, using a single 0.1-ml sample from each clear tube for subculture, and adopting an alternate method for calculating endpoint determination. In order to test these recommendations in a clinical laboratory setting, we used the modified methodology on 224 separate tests for bactericidal activity. There were 102 serum bactericidal titer (SBT) and 122 minimum bactericidal concentration (MBC) assays performed. By defining reproducibility as agreement between duplicate tests ± 1 dilution, we found 207 of 224 tests (92%) were reproducible at the 24-h subculture point (94% for the SBT assay and 91% for the MBC assay). When the 17 assays with discrepant results were incubated an additional 24 h for a second subculture, only 1 of 224 tests (0.4%) remained discrepant. The method used is practical for a clinical laboratory that chooses to perform bactericidal activity testing and assures a high level of reproducibility between duplicate assays. The total cost of a test was approximately $25.00.

With the emergence of multidrug-resistant organisms, physicians are now faced with the challenge of treating infections that have no established therapeutic guidelines (2). Under these circumstances, they may request that the clinical laboratory attempt to assess the adequacy of therapy (5, 9, 23). Bactericidal activity testing has been used as an occasional guide for antimicrobial agent treatment since the 1950s. Such testing is most frequently performed when bactericidal antimicrobial agent therapy is considered necessary (23). The two most commonly used of these special microbiology tests to monitor potentially nonstandard therapy are those that determine the minimum bactericidal concentration (MBC) of a drug and the serum bactericidal titer (SBT) of a patient’s blood or body fluid during treatment (23). Reference guidelines from the National Committee for Clinical Laboratory Standards (NCCLS) are now approved for performing both of these assays (16, 17). However, several important variables affect the reproducibility of the test results (23), and a recent assessment by MacGowan and coworkers again highlighted continued technical problems (12). Since neither the new NCCLS documents nor any recent published information comments on how various methodologies actually perform during clinical laboratory use, reproducibility for both the MBC and SBT assays still needs to be addressed (11, 13).

Between April 1992 and November 1997, the microbiology laboratory at Northwestern Memorial Hospital used a procedure that combines the NCCLS method(s) with that described by Peterson and Shanholtzer (16, 17, 23). The purpose of this report is to summarize our experience with this modified methodology by using results from the clinical laboratory tests.

MATERIALS AND METHODS

The MBC and SBT tests are performed by using similar methodologies, with minor exceptions, as described below.

Test strategy. To prepare for testing, it is necessary to determine the 6 dilutions that will be run in duplicate for the MBC test. The MIC was first determined by NCCLS reference agar dilution methodology (15), and then 4 dilutions above the MIC, and 1 dilution below the MIC were used for the levels to be assessed in the MBC assay. Running 4 dilutions above the known MIC permits detection of tolerance to normally bactericidal agents (23). For SBT testing, the 6 twofold dilutions are constant: 1:2 through 1:512 for both peak and trough levels. These dilutions were considered sufficient based upon the NCCLS reference method for SBT testing, which defines titer determinations up to 1:32 for interpretation of test results (16). A positive and negative growth control tube is included with each test. Quality control is also performed with a known reference organism each time a fresh antimicrobial agent stock solution is prepared for MBC testing.

Dilution preparation. Antimicrobial agents used for MBC testing are obtained from the drug’s manufacturer as reference powder or solution. Stock solutions at 1,000 μg/ml are freshly prepared the day of use or are prepared from a frozen stock solution stored at −70°C. Serial dilutions for both test methods are prepared in borosilicate glass test tubes using cation-adjusted Mueller-Hinton broth (CAMHB, Difco Laboratories, Detroit, Mich.). For the MBC test, the duplicate set of tubes is prepared by filling each tube with 1 ml of CAMHB, beginning with tube 2. Next, 1 ml of CAMHB containing twice the desired highest drug concentration is added to tubes 1 and 2. Tube 2 is then vortexed, and 1 ml of the mixture is removed and added to tube 3. Tube 3 is vortexed, and 1 ml of this mixture is removed with a new pipette and transferred to tube 4. This process is continued until the last tube is reached. One ml is discarded from the last tube. To bring the tubes to a final volume of 2 ml, 1 ml of CAMHB is added to each tube. The growth and sterility controls are prepared by adding 2 ml of CAMHB to each of two new test tubes.

For the SBT test, six tubes, in duplicate, are filled with 1 ml of CAMHB, again beginning with tube 2. One ml of the patient’s serum is added to tubes 1 and 2, and tube 2 is vortexed. One ml of the mixture is removed from tube 2, added to tube 3, and vortexed, and the process is continued until the last tube is reached. One ml of the mixture is discarded from the last tube. Finally, 1 ml of CAMHB
Enterococcus faecalis
Penicillins, ampicillin-sulbactam, gentamicin, teicoplanin, imipenem

Enterococcus faecalis
Vancomycin, ampicillin-sulbactam, gentamicin, teicoplanin, rifampin

Pseudomonas aeruginosa
β-Lactam antibiotics (penicillins and cephalosporins), ciprofloxacin, aminoglycosides, polymyxin B

Beta-hemolytic streptococci
Penicillins, gentamicin, clindamycin

Viridans streptococci
β-Lactam antibiotics (penicillins and cephalosporins), gentamicin, vancomycin

TABLE 1. Summary of organism-antimicrobial agent combinations tested

<table>
<thead>
<tr>
<th>Organism</th>
<th>Antimicrobial agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>Glycopeptides, aminoglycosides, cephalosporins, rifampin, oxacillin, clindamycin, trimethoprim-sulfamethoxazole, levofloxacin</td>
</tr>
<tr>
<td>MRSA</td>
<td>Vancomycin, gentamicin, rifampin</td>
</tr>
<tr>
<td>Coagulase-negative staphylococci</td>
<td>Trimethoprim-sulfamethoxazole, oxacillin, aminoglycosides, vancomycin, rifampin</td>
</tr>
</tbody>
</table>

is added to each tube to bring the final volume to 2 ml. Growth and sterility tubes are prepared in the same manner as indicated for the MBC test.

Inoculum and incubation. Four to five 18- to 24-h colonies are inoculated into 3 ml of Trypticase soy broth. The broth is incubated for 3 to 5 h to achieve a turbid suspension. The inoculum is prepared by adjusting the logarithmic-phase growth to match the turbidity of a 0.5 McFarland standard, yielding approximately 10^4 CFU/ml. The suspension is then diluted 1:10 with CAMHB to give a working inoculum of 10^3 CFU/ml. Using a calibrated pipette, 0.1 ml of the working inoculum is carefully added to each tube, except the sterility control, just below the broth surface to produce a final density of approximately 5 × 10^5 CFU/ml. Mixing is performed in the tube by gently flushing the inoculum in and out of the pipette tip four or five times, avoiding splashing or creation of bubbles. All tubes are incubated at 35°C for 24 h without shaking or agitation. A colony count is performed on the starting test inoculum by lawning 0.01 ml of a 100-fold dilution of the growth control tube contents onto a drug-free blood agar plate to determine if the estimated organism density for the test is within the actual desired limits.

Determining endpoints. At the end of 24 h of incubation, the tubes are read for the MIC or serum inhibitory titer, defined as the concentration of the first tube in the series (ascending drug concentration or descending serum dilution titer) to show no visible trace of growth. The MBC or SBT is then determined by sampling the macroscopically clear tubes and the first turbid tube in the series. Before being sampled, the tubes are gently mixed by shaking them with a pipette, and a 100-μl aliquot is removed. Each aliquot is placed on a single antibiotic-free agar plate suitable for the growth of the microbe being tested, in a single streak down the center of the plate (24). The sample is allowed to be absorbed into the agar until the plate surface appears dry (about 30 min). The aliquot is then spread over the plate by using a lawning technique. This subculture method in the Northwestern Memorial Hospital clinical microbiology laboratory showed 99.6% reproducibility when applied to 224 tests. The modification of the NCCLS procedures consists of deletion of serum additives, selecting four to five

TABLE 2. Results of bactericidal activity testing by the modified procedure

<table>
<thead>
<tr>
<th>Test</th>
<th>No. of tests performed</th>
<th>Duplicate agreement (%) after 24-h incubation</th>
<th>Duplicate agreement (%) after 48-h incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBC</td>
<td>122</td>
<td>111 (91)</td>
<td>121 (99)</td>
</tr>
<tr>
<td>SBT</td>
<td>102</td>
<td>96 (94)</td>
<td>102 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>224</td>
<td>207 (92)</td>
<td>223 (99.6)</td>
</tr>
</tbody>
</table>

DISCUSSION

The results of testing by using the suggested bactericidal test method in the Northwestern Memorial Hospital clinical microbiology laboratory showed 99.6% reproducibility when applied to 224 tests. The modification of the NCCLS procedures consists of deletion of serum additives, selecting four to five
colonies to prepare the inoculum, avoiding agitation during incubation, performing the test in duplicate with six dilutions, using a larger subculture volume from the incubated tubes, reincubating for 24 h to resolve discrepancies, and using an alternate statistical endpoint determination.

Enhanced clinical relevance has not been shown for serum additives in tests of virtually all currently used antimicrobial agents; therefore, they are not recommended in order to reduce the risk of transmission of blood-borne pathogens. We recognize that the influence of protein in the in vitro activity of antibiotics can be significant (22) and that protein binding affects interpretation of serum kinetics and extravascular drug penetration (18). However, many factors affect the in vivo binding of drugs to serum proteins (25). Seriously ill patients, the group most likely to have the testing described in this report performed, are very likely to have abnormal serum protein binding in vivo. Chen and colleagues found that serum albumin additives from commercial sources that may be used for MBC and SBT testing have unpredictable factors that affect binding of cell wall-active agents (4). We have also shown that filter membranes for preparation of serum ultrafiltrates can trap antibiotics (20), and this phenomenon will markedly alter the tubes is not necessary prior to sampling due to the 0.1-ml size of the initial inoculum. Taylor et al. evaluated the effect of inoculum volume on the outcome of macrodilution tests (26). They found that gentle agitation at 20 h was needed only when a large volume of inoculum (1.0 ml) was used; however, it was not useful when the 0.1-ml inoculum volume we suggest was adopted (26). Gresser-Burns and colleagues (8) and Ishida et al. (10) have also found it preferable to use a smaller inoculum (0.1 ml) and not to disturb the sample until the time of the actual subculture.

Testing in duplicate is recommended, since bactericidal activity tests historically have shown poor reproducibility. By reincubating the tubes after the first 24 h of incubation, one can attempt to resolve discrepancies (such as the skipping phenomenon or endpoint discrepancies) between duplicates without needing to repeat the entire test. Cell wall-active agents like β-lactams and vancomycin kill slowly, a phenomenon discussed in the new NCCLS documents (16, 17). Therefore, it is not unexpected that occasional isolates will not have fully responded to the killing activity of the drug being tested at 24 h, thus leading to a discrepancy in duplicate test results if sampling is done at a time when colony numbers are undergoing a rapid decline. The additional 24 h, then, allows for enhanced reproducibility of an assessment of the agent’s potential bactericidal activity when its bactericidal action is complete and the number of viable colonies is more stable.

Sampling a larger volume (0.1 ml) from the macrodilution tubes and using the statistical correction formula \( (n + 2\sqrt{n}) \) for bactericidal activity endpoint determination provides a simple method for determining 99.9% killing that includes 95% confidence limits. The larger sampling volume, 0.1 ml instead of 10 \( \mu l \), provides a greater number of colonies to be counted (10 to 100 versus 1 to 10), theoretically enhancing test precision, particularly if the initial test inoculum approaches the lower recommended test limit of \( 10^5 \) CFU/ml.

The method described for determining bactericidal activity has improved reproducibility and, presumably, reliability of this test without increasing the workload for our laboratory.

### TABLE 3. Summary of 17 discrepancies found at 24 h of incubation after first subculture

<table>
<thead>
<tr>
<th>Organism</th>
<th>Antimicrobial agent(s)</th>
<th>Test type</th>
<th>Discrepancy</th>
<th>Resolved with additional incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin-resistant Enterococcus faecalis</td>
<td>Ampicillin</td>
<td>SBT</td>
<td>Skipping</td>
<td>Yes</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Aminoglycoside</td>
<td>SBT</td>
<td>Paired SBT results did not agree</td>
<td>Yes</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>Ampicillin</td>
<td>MBC</td>
<td>Skipping</td>
<td>Yes</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Oxacinil</td>
<td>SBT</td>
<td>Paired SBT results did not agree</td>
<td>Yes</td>
</tr>
<tr>
<td>Staphylococcus haemolyticus</td>
<td>Vancomycin</td>
<td>MBC</td>
<td>Paired MBC results did not agree</td>
<td>Yes</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Oxacinil</td>
<td>MBC</td>
<td>Paired MBC results did not agree</td>
<td>Yes</td>
</tr>
<tr>
<td>Streptococcus anginosus</td>
<td>Penicillin</td>
<td>SBT</td>
<td>Poor growth of organism at 24 h</td>
<td>Yes</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Oxacinil</td>
<td>MBC</td>
<td>Paired MBC results did not agree</td>
<td>Yes</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Vancomycin</td>
<td>SBT</td>
<td>Paired SBT results did not agree</td>
<td>Yes</td>
</tr>
<tr>
<td>MRSA*</td>
<td>Vancomycin</td>
<td>MBC</td>
<td>Skipping</td>
<td>Yes</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Gentamicin</td>
<td>MBC</td>
<td>Paired MBC results did not agree</td>
<td>No</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>Ampicillin, streptomycin, rifampin, and vancomycin</td>
<td>SBT</td>
<td>Paired SBT results did not agree</td>
<td>Yes</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>Ampicillin, streptomycin, rifampin, and vancomycin</td>
<td>SBT</td>
<td>Skipping</td>
<td>Yes</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Aztreonam</td>
<td>MIC and MBC</td>
<td>Paired MIC and MBC results did not agree</td>
<td>Yes</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Cefazolin</td>
<td>MIC and MBC</td>
<td>Paired MIC results did not agree</td>
<td>Yes</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Oxacinil</td>
<td>MIC and MBC</td>
<td>Paired MIC and MBC results did not agree</td>
<td>Yes</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Vancomycin</td>
<td>MBC</td>
<td>Skipping</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*MRSA: methicillin-resistant Staphylococcus aureus.
This assessment of our current laboratory practice builds upon work we had previously published on bactericidal testing techniques. The report by Shanholtzer and colleagues had shown that macrodilution bactericidal tests were not reproducible when simultaneously performed by two workers or if performed multiple times by one worker when using methodology that included the NCCLS currently recommended agitation step at 22 to 24 h and a subculture volume of 0.01 ml (24). The subsequent report by Gresser-Burns et al., using an MBC subculture volume of 0.1 ml, no agitation during incubation, and the n + 2\sqrt{n} endpoint calculation, demonstrated resolution of the skipping phenomenon when incubation was extended from 24 to 48 h (8). They found that growth at higher drug concentrations (above a concentration with no growth at 24 h of subculture) disappeared when using the 48-h incubation time point in eight of nine tests (8). Several different medical technologists have performed our current technique over an 8-year period in the clinical laboratory with no change in outcome reproducibility. The cost of $25.00 per test is not inexpensive, but it is modest when one realizes that the total hospitalization expense per day for a seriously ill patient is now approximately $2,000. This modified method can be easily performed in any clinical microbiology laboratory and provides a high level of test reproducibility.

ACKNOWLEDGMENT

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