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Variation in Performance of the Serum Bactericidal Test

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Questionnaires were sent to 37 diagnostic microbiology laboratories to assess their methods for performance of the serum bactericidal test. Of the 28 responding laboratories, 26 utilized the test, with considerable variation among methods. Culture and dilution media included Trypticase soy, Mueller-Hinton, brain heart infusion, Columbia broth, dextrose phosphate, and pooled human serum. Several different schemes for dilution of the test serum were employed, including both micro and macro methods. The greatest variation occurred in the size of the bacterial inoculum, with only eight laboratories having a method for standardization of the inoculum. Other differences included the time of incubation, the method of subculture, and determination of the bactericidal end point. These studies emphasize the need for assessment of the variables in the performance of this test and for the development of a standard method for diagnostic laboratories.

In 1947, Schlichter and MacLean (12) suggested a useful guide for effective antibiotic therapy in the patient with subacute bacterial endocarditis. This consisted of measuring the bacteriostatic effect of serial dilutions of the patient's serum against his own infecting organism during antibiotic therapy. This test of antibacterial activity was modified by several investigators, including Jawetz (9), to measure bactericidal effect.

In recent years, requests for this test have been increasing at many medical centers; approximately 200 serum bactericidal levels now are performed yearly in our laboratory. As part of our reevaluation of both laboratory and clinical aspects of this test, the literature was reviewed and it reflected a marked variation in the techniques employed by different laboratories. To assess the present degree of variation in the performance of this test, a questionnaire was prepared and submitted to the directors of 37 diagnostic microbiology laboratories. The results of this survey form the basis of this presentation and emphasize the great variation in the performance of this test.

MATERIALS AND METHODS

Detailed questionnaires were sent in Otober 1973 to 37 diagnostic microbiology laboratories representing most geographic areas in the United States. Thirty of these laboratories were at medical school centers. Twenty-eight laboratories responded, including two that did not perform the serum bactericidal test (SBT).

RESULTS

General comments. The number of tests performed ranged from 2 to 250 per year. As would be expected, the SBT was done with highest frequency in the major teaching centers. However, 17 of 26 laboratories performed less than 75 tests per year. The leading organism employed in the SBT was *Staphylococcus aureus*. The next most common organisms were *Streptococcus* sp. of the viridans group, *Streptococcus* sp. of the enterococcal group, *Pseudomonas* sp., and *Escherichia coli*.

Culture and dilution media. Most laboratories used the same medium for growth of the inoculum and for dilution of the test serum. However, three laboratories employed Trypticase soy for preparation of their inoculum and Mueller-Hinton or brain heart infusion broth for dilution of the test serum. The various culture media employed by these laboratories are shown in Table 1. Trypticase soy was the most common broth used to grow the patient's infecting organism. Mueller-Hinton and brain heart infusion broth were also commonly employed. Fifteen of the twenty-six laboratories prepared the inoculum by incubation for 18 to 24 h, ten by incubation for 3 to 6 h, and one by incubation long enough to obtain a standard optical density. The most common media used for preparation of the serum dilutions are also shown in Table 1. Again, Trypticase soy was the most common. Only two laboratories prepared their dilutions with pooled human serum.

TABLE 1. *Culture media employed in the SBT*

Liquid media	No. of responding laboratories employing:	
	Culture medium	Dilution medium
Trypticase soy	17	12
Mueller-Hinton	3	5
Brain heart infusion	4	5
Columbia	1	1
Dextrose phosphate	1	1
Pooled human serum		2

Dilution schemes. Significant variation occurred in preparation of the dilutions of the test serum. Only two laboratories used 2.5 ml as their total final volume, as suggested by Jawetz (9). Fifteen employed a final volume of 1 ml; however, in one-fourth of these, this included a rather large bacterial inoculum of 0.4 to 0.5 ml. Two laboratories used very small total volumes of 0.10 to 0.20 ml with microtiter methods. One-half of the laboratories started at an initial 1:2 dilution of the patient's serum. The remainder tested the serum undiluted or at initial dilutions of 1:4 or 1:5.

Bacterial inoculum size. The greatest variable in performance of the test was the inoculum size of the bacterium. Only eight of the laboratories standardized the inoculum size and these were done in a number of different ways (Table 2). The great diversity of nonstandardized inoculum size, which differed as much as 1,000-fold in the final inoculum concentration, is shown in Table 3.

Determination of bactericidal effect. Although the majority of laboratories used subculture to blood agar as the principal method for detecting bactericidal effect, six employed a variety of media including Mueller-Hinton, Trypticase soy, brain heart infusion, and thioglycolate broth. The amount of fluid inoculated onto the subculture media differed widely. Sixteen laboratories used calibrated loops for inoculation in sizes of 0.005, 0.001, 0.03, and 0.01 ml. Six laboratories used one loopful from noncalibrated loops. Four institutions pipetted from 0.05 to 1.0 ml onto subculture media. The portion of an agar plate used for subculture ranged from $\frac{1}{10}$ to $\frac{1}{2}$ of a plate, with the most common amounts $\frac{1}{4}$ and $\frac{1}{8}$. All but one laboratory incubated their subcultures for 18 to 24 h; the only exception was one that incubated their subcultures for 48 h. The number of laboratories and the number of colonies each allowed in the determination of the bactericidal end point is as follows, respectively: 5, 0; 3, 1; 4, 5; 7, 10; 1, 50; 1, 10^2 reduction; 2, 10^3 reduction; and 1, 0 and 10^3 reduction. A wide range of allowable bacterial growth among the individual laboratories is evident.

DISCUSSION

The SBT is an accepted method for assessing the efficacy of treatment in bacterial endocarditis (7, 12, 13). It has been recommended for other clinical conditions such as osteomyelitis (8), staphylococcal pneumona (8), meningitis (13), and in modified form for tuberculosis (3). The present study emphasizes the variability in the performance of this test and the need for careful evaluation of the conditions for its optimal performance.

Several factors in the measurement of the serum bactericidal level have been discussed previously in literature. Jawetz (9) mentions the

TABLE 2. *Bacterial inoculum size (standardized)*

Method	No. of laboratories	Inoculum size[a]	Final dilution vol (ml)	Final inoculum concn (organisms per ml)[b]
Nephelometry	2	5×10^2	1.0	5×10^2
		NS	0.1	
Turbidity standards	3	10^6	0.6	1.7×10^6
		3×10^7	1.0	3×10^7
		2×10^6	1.0	2×10^6
Colony counts	2	10^5	2.5	4×10^4
		10^3	2.0	5×10^2
Unspecified	1	10^5	1.0	10^5

[a] Approximate number of organisms; NS, not stated.
[b] Approximate.

TABLE 3. *Bacterial inoculum size (nonstandardized)*

Lab no.	Incubation time of inoculum	Bacterial dilution	Inoculum vol (ml)	Final test vol (ml)
1	Overnight	10^{-5}	0.5	1.0
2	Overnight	10^{-5}	1.0	1.0
3	Overnight	10^{-4}	0.4	1.0
4	Overnight	10^{-4}	0.5	1.0
5	Overnight	10^{-4}	0.5	1.0
6	4 to 6 h	10^{-4}	0.5	1.0
7	Overnight	10^{-3}	0.01	0.51
8	4 h	10^{-3}	0.05	1.0
9	Overnight	2×10^{-3}	0.05	0.2
10	3 h	10^{-3}	0.1	1.0
11	Overnight	10^{-3}	0.5	1.0
12	Overnight	10^{-3}	0.5	0.5
13	Overnight	10^{-3}	0.5	1.0
14	Overnight	10^{-3}	1.0	2.0
15	Overnight	10^{-2}	0.05	2.0
16	Overnight	10^{-2}	0.1	2.5
17	Overnight	10^{-2}	0.5	1.0
18	6 h	10^{-2}	1.0	2.0

critical factor of inoculum size. An unduly large amount of bacteria may result in a misleadingly low estimate of serum bactericidal activity, whereas a very small inoculum may produce a misleadingly high value. In our survey of diagnostic microbiology laboratories, the inoculum size was the factor of greatest variability. Two-thirds of the laboratories did not employ a standardized inoculum. Of the institutions that counted the bacteria, the number of organisms differed by as much as 1,000-fold. This variation in inoculum size would be expected to produce different SBT results, which, therefore, may affect the physician's choice of antibiotic regimen. In 1971, the International Collaborative Study of Antibiotic Sensitivity Testing recommended an inoculum size of 10^5 to 10^6 colonies per ml (6). They recommended also that 1 ml of inoculum be added to each dilution tube with a final volume of 2 ml. A similar scheme could be employed in an attempt to standardize the SBT procedure.

Another factor in the performance of the serum bactericidal test is the choice of media. Different culture media have been noted to alter broth dilution bacteriostatic determinations (6), although this has not been studied with bactericidal tests. In our survey, a majority of the laboratories employed Trypticase soy broth, although four other types of broth were also used. It would seem reasonable in the standardizing of the SBT procedure to grow all organisms without special growth requirements in Mueller-Hinton broth, the recommended medium for in vitro susceptibility testing (6). Kunin states that the broth-dilution assay for serum antibacterial activity may be falsely elevated for those antibiotics which have high protein binding (11). He suggests that, in these instances, dilutions should be done with human serum instead of broth. However, only 2 of the 26 responding laboratories used human serum in their SBT dilutions. Changes in the end point of the SBT, as a result of using serum rather than broth to prepare dilutions of the test serum, have not been studied extensively. Such an assessment is being performed currently in our laboratory.

Correlating the results of the SBT with the in vivo effectiveness of the tested antibiotic poses an additional major problem, since the conditions in the test tube bear little resemblance to the complex environment encountered within the patient. Most laboratories, in the performance of the SBT, incubated dilutions of the patient's serum with his infecting organism for 18 to 24 h. However, in most individuals, peak levels of serum antibiotic activity last for a shorter period of time, depending on the frequency of antibiotic administration, the pharmacokinetics of the particular drug, and the patient's own ability to excrete or metabolize the drug. The importance of such potential differences is emphasized by the observations of Eagle et al., that there are marked differences among bacterial species and occasional differences among different strains of the same species with respect to the maximal rate at which they could be killed by penicillin G, both in vivo and in vitro (4, 5). In addition, Jawetz et al. (10)

have pointed out that, in vivo, the bacteria may be in a low dormant state of metabolic activity, often intracellular or in a localized walled off abscess, and growing in a necrotic, complex chemical milieu which may inactivate the antibiotic. Finally, the SBT does not measure other antibacterial defenses of the patient, such as his natural serum bactericidal complement-dependent system, serum antibodies, phagocytosis, etc., which may participate with the antibiotic in controlling and irradicating infection.

Consideration must also be given to the possibility that alterations in the chemical properties of the tested antibiotic may occur which will affect its biological activity. These could reflect differences in the performance conditions of SBT as compared with those encountered in the patient. For example, chlortetracycline looses 94% of its biological activity when stored in normal saline at 37 C for 18 h (14); colistin undergoes chemical activation in serum at 37 C so that antibacterial activity after 18 h of incubation is fourfold higher than with a 3-h incubation technique (1); and clindamycin phosphate has no in vitro activity in broth and requires prior hydrolysis in serum for antibacterial activity (2).

Ultimately, the validity for any method of performing the SBT depends on its ability to accurately predict the clinical response to a given infection. The present failure to perform the SBT in a standardized manner suggests that it is necessary to assess the clinical usefulness of the test as it is performed in each laboratory. Perhaps a more direct approach would be an attempt to standardize the conditions for performance of this test to more rapidly accumulate information on its overall usefulness in specific clinical situations. This would prove particularly valuable to those laboratories who do not perform this test frequently. Toward this goal, we are currently investigating some of the variables in performance of this test and are assessing our clinical experience in patients who have been monitored with the SBT.

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