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Comparison of a Micromethod for Performance of the Serum Bactericidal Test with the Standard Tube Dilution Method

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A micromethod for performance of the serum bactericidal test is described, and the results obtained with this method are compared with those obtained with the standard tube dilution macromethod. An agreement within ± 1 dilution was achieved in 23 of 25 (92%) determinations of the serum bactericidal titer. The micromethod used approximately one-third of the amount of pooled normal human serum and of the technician's time required for performance of the macromethod. The micromethod offers an accurate and economical alternative to the macromethod for the performance of the serum bactericidal test and is particularly useful with infants and children since it minimizes blood loss.

The principle of the serum bactericidal test was first described by Schlicter and MacLean in 1947 (7). The test provides a direct measurement of the antimicrobial activity of a patient's serum against the patient's infecting organism. In addition to monitoring antimicrobial therapy in patients with infective endocarditis, this test has been used to adjust therapy in patients with cancer with bacteriologically proven infections (4), in patients with staphylococcal pneumonia (3) and meningitis (8), and most recently in children with acute hematogenous osteomyelitis or septic arthritis (10; C.G. Prober and A.S. Yeager, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 18th, Atlanta, Ga., Abstr. no. 189, 1978).

The important variables in standardizing the macromethod for the performance of the serum bactericidal test have been reported previously (5, 6). The present study describes a micromethod for the performance of this test which reduces the amount of normal human serum required as a diluent, is simple and efficient to perform, and reduces blood loss to the patient. An intralaboratory comparison of the micro- and macromethods for determination of the serum bactericidal level in patients' sera has not been reported previously and is included in this study.

MATERIALS AND METHODS

Serum. The serum bactericidal titers (SBTs) were measured by macro- and micromethods in our diagnostic laboratory on 26 samples of serum obtained from 11 patients with documented systemic infections (Table 1). The blood was allowed to clot at room temperature, and after centrifuging the serum was removed and assayed immediately or frozen at −20°C.

Bacteria. The patients' infecting organisms were used in assessing the bactericidal activity of their own serum. The final inoculum was taken from a 10^{-3} dilution of an 18- to 24-h growth of the test organism in Mueller-Hinton broth (1). On 16 of 16 occasions, this dilution was shown to result in a concentration that fell within the expected range of 4×10^5 to 1×10^6 organisms per ml. Because the final volume of the inoculum in the macromethod was 0.5 ml, this represented 2×10^5 to 5×10^5 bacteria. In the micromethod the final inoculum was one log lower.

Macromethod. This test was performed as described previously (6). A 0.5-ml amount of heat-inactivated (56°C for 30 min), pooled normal human serum was added to tubes 2 through 10 and 0.5 ml of the patient's serum was added to each of the first two tubes. Serial twofold dilutions were made beginning with the second tube, leaving a final volume of 0.5 ml in each tube. A 0.5-ml amount of the 10^{-3} dilution of the 18- to 24-h culture of the patient's organism in Mueller-Hinton broth was added to each tube, giving a final volume of 1.0 ml and a final concentration of organisms of 2×10^5 to 5×10^5. The range of dilutions of the patient's serum was 1:2 to 1:1,024. After incubation for 18 to 24 h at 35°C, 0.01 ml of the mixture from the tubes with no visible growth was picked up with a calibrated platinum loop and spread onto sections of a blood agar plate. The plate was incubated for 18 to 24 h at 35°C. A growth of 10 colonies or less on this subculture was considered "bactericidal" and represented 99.5% or greater killing of the initial inoculum. The last tube showing such a result was recorded as the SBT.

Micromethod. The test was modified from a micromethod reported by Stratton and Reller (9). Microtiter plates (Micro-Media Systems Co., San Jose, Calif.) were used for the tests. The test was always performed in triplicate. A 0.05-ml amount of heat-inactivated (56°C for 30 min), pooled normal human

TABLE 1. *Comparison of the SBTs determined by the macro- and micromethods*

Patient	Organism	Source	Antibiotic	Macro-SBT	Micro-SBT
1	*Staphylococcus* (coagulase +)	Hip	Dicloxacillin	<1:2	<1:2
				<1:2	<1:2
2	*Staphylococcus* (coagulase +)	Hip	Nafcillin	1:32	1:128
				1:128	1:128
3	*Staphylococcus* (coagulase +)	Hip	Cefoxitin	1:2	1:2
				1:8	1:16
				1:16	1:4
				1:16	—[a]
4	*Escherichia coli*	Blood	Gentamicin	1:4	1:4
			Cefazolin	1:4	1:4
5	*Escherichia coli*	Blood	Gentamicin	1:8	1:16
			Cefazolin	1:16	1:16
			Carbenicillin		
6	*Escherichia coli*	Blood	Gentamicin	1:32	1:32
				1:32	1:32
			Chloramphenicol	1:64	1:64
				1:64	1:64
7	*Enterobacter cloacae*	Urine	Gentamicin	1:4	1:8
			Nafcillin	1:32	1:32
8	*Klebsiella pneumoniae*	Intra-abdominal abscess	Gentamicin, keflin	1:8	1:8
			Amikacin, clindamycin, keflin	1:64	1:32
9	*Pseudomonus aeruginosa*	Sputum	Gentamicin	<1:4	<1:4
			Cefazolin	<1:2	<1:2
10	*Serratia marcescens*	Blood	Gentamicin	<1:2	<1:2
				1:8	1:8
				1:16	1:16
11	*Serratia marcescens*	Chest tube drainage	Tobramycin, cefazolin	<1:2	<1:2

[a] Triplicates values did not allow the assignment of a titer; see text.

serum was added to each well of columns 2 through 10 in each row. Then, 0.1 ml of the patient's serum was added to the first well of each row, and serial twofold dilutions of the serum were produced with 0.05-ml Microdiluters (Cooke Engineering Co., Alexandria, Va.). A 0.05-ml quantity of a 10^{-3} dilution of the 18- to 24-h culture of the patient's organism in Mueller-Hinton broth was added to each well with a disposable calibrated dropping pipette (Dynatech Laboratories Inc., Alexandria, Va.), giving a final volume of 0.1 ml. The range of dilutions of the patient's serum was 1:2 to 1:1,024. The contents of the trays were mixed carefully by vibrating the tray on a board placed on top of a Vortex mixer, covered, and placed in a closed humid incubator for 18 to 24 h at 35°C. After this incubation period, a second microtiter tray containing 0.1 ml of Mueller-Hinton broth in each well was prepared, and an 80-pronged inoculator was used to transfer 0.005 ml from each well of the initial tray to each respective well of the second tray. Before sampling for transfer, the initial tray was mixed as described above. The second tray was covered, placed in a closed humid container, and incubated for 18 to 24 h at 35°C. After this incubation period, the tray was examined on a light base with a dark-field background. The highest dilution of the patient's serum showing no turbidity, or button of growth, or both was defined as the SBT and represented greater than 99.9% killing of the initial inoculum. Each of the triplicate titers was recorded, and the final titer was derived as follows. If all three agreed, this was the titer; if two of the three agreed, the third was disregarded; and if all three disagreed but were within one well of each other, the median was used. With any other variation, the test was repeated.

For both the macro- and micromethods, portions of the pooled normal human serum and of Mueller-Hinton broth were included as tests of their sterility. The purity of the bacterial inoculum was confirmed by streaking a portion of the final dilution onto a blood agar plate and examining this plate after 18 to 24 h of incubation at 35°C.

RESULTS

When the micromethod was used, there was concordance between two or more of the replicate tests on 24 of 26 (92%) occasions. All three replicates were the same on 12 of 26 (46%) occasions; two of the three were the same on 12 of 26 (46%) occasions; all differed on 1 of 26 (4%) occasions; and no determination could be made on only 1 of 26 (4%) occasions.

The SBT obtained with the micromethod was the same as that obtained with the macromethod on 19 of 25 (76%) occasions (Table 1). The SBT was higher with the micromethod than the macromethod on four occasions and lower on two occasions. The results were identical or within one dilution of each other on 23 of 25

(92%) occasions, and all results were within two dilutions of each other.

DISCUSSION

A micromethod for the measurement of the SBT has been defined, and the results of its application have been compared with those obtained by the standard tube dilution method. Of the SBTs determined by either the micro- and macromethods, 92% were the same or within ±1 dilution of each other. This is about the same degree of correlation which was found when the microtiter method was compared with the tube dilution method for determination of the minimal inhibitory concentration (2).

The variations in the performance of the serum bactericidal test in different laboratories have been discussed previously (5). In the present study, the same reagents were used in both tests and included pooled normal human serum as the diluent since we have previously shown that the use of broth significantly alters the titers in sera obtained from patients receiving highly protein-bound drugs (6). Although the bacterial inoculum in the micromethod was 1/10th of that used in the macromethod, the final concentration of the organisms in the tubes and the wells was the same.

The micromethod had several important advantages over the macromethod. The same technician could perform the micromethod in triplicate in approximately one-third of the time required to perform the macromethod. Equally important was the requirement of only 1.35 ml of pooled human serum to set up the micromethod in triplicate as compared with 4.5 ml for a single determination with the macromethod. Finally, the small amount of the patient's serum required for the micromethod has distinct advantages when studying infants and children.

On the basis of the results of our studies, the micromethod has been adopted as the standard method in our diagnostic laboratory. As our experience with this test has increased, difficulty has been encountered in determining the visual endpoint with occasional organisms which were slow growing or fastidious in their growth requirements. For these organisms, the subcultures to assess the bactericidal endpoint have been modified as follows. After the contents of the tray were mixed by vibration, the inoculator was dipped into the tray and 0.005 ml was transfered to the surface of a Mueller-Hinton agar plate, which with some organisms included 5% sheep erythrocytes. Surface growth on agar rather than growth in broth was used because of difficulty in obtaining a clear visual endpoint in broth with slow-growing organisms. Care was taken to gently but firmly press down on the transfer prongs to insure delivery of the sample from each prong. The Mueller-Hinton agar plate was prepared in the covering lid that comes with the micromedia plate. After transfer, the plate was covered loosely with Parafilm and incubated Parafilm down, for 18 to 24 hours at 35°C. The endpoint was read as the last well showing no growth on subculture and avoided errors in a numerical endpoint that might result from failure to adequately separate individual colonies in the small area that was inoculated.

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