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Serum Bactericidal Testing with the Autobac System

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Current methodology for the serum bactericidal test requires a minimum of 48 h. A procedure was devised for performing this test with the Autobac system (General Diagnostics, Div. Organon Inc., Raleigh, N.C.) in a shortened time span. All titers obtained with the Autobac were compared against results obtained with a standardized tube dilution procedure. The Autobac low-thymidine eugonic broth performed comparably to the tube dilution diluent, a 1:1 ratio of pooled human serum and cation-supplemented Mueller-Hinton broth (99.2% correlation between bactericidal endpoints). Over 300 tests were conducted by using stock reference bacterial strains, clinical isolates, pooled human serum seeded with antimicrobial agents, and serum from patients on antimicrobial therapy. With the Autobac procedure, serum inhibitory titers can be reported in 3 to 4 h (93.4% correlation with the tube dilution procedure). Serum bactericidal titers can be obtained in 24 h without the necessity of subculturing (95.6% correlation). With the exception of staphylococci tested against penicillin, serum bactericidal titers can be obtained in 3 to 4 h (88.4% correlation). The Autobac procedure can provide the clinical laboratory with a rapid, reliable method for performing the serum bactericidal test.

During the past decade, much progress has been made in adapting microbiological procedures to enable rapid reporting of results. Automated and semiautomated instrumentation is now available that can report susceptibility testing results, as well as organism identification, in as little as 3 to 5 h. Fluoroimmunoassay techniques have been developed which can supply antimicrobial assay results in 15 min (6). However, little work has been done to shorten the length of time required to perform the serum bactericidal or Schlichter test, a procedure which involves a minimum of 48 h of incubation.

A procedure which could provide more rapid results for the serum bactericidal test might yield other advantages. Intermittent antimicrobial therapy results in peak body fluid levels in 1 to 2.5 h (2); a shorter incubation period may more closely reflect the in vivo exposure of microorganisms to the therapeutic agent. Shorter incubation also lessens the likelihood of pH and nutritional changes in the culture medium, which, in turn, may affect the synthesis of bacterial inactivating enzymes.

This study was undertaken to provide a procedure for performing the serum bactericidal test with the Autobac system and to determine the minimum amount of time required to report results of both bacteriostatic and bactericidal serum levels.

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MATERIALS AND METHODS

Microorganisms. American Type Culture Collection strains were obtained from Difco Laboratories (Detroit, Mich.); two strains (*Escherichia coli* 29194 and *Staphylococcus epidermidis* 29885) were supplied by General Diagnostics, Div. Organon Inc., Raleigh, N.C. Clinical isolates were selected from specimens submitted to the microbiology laboratory of the Cleveland Clinic Foundation, Cleveland, Ohio.

Pooled human serum. The pooled human serum was obtained from the Cleveland Clinic Foundation serology department. Each aliquot was assayed for antimicrobial activity by a disk diffusion assay technique (3). Antimicrobial agent-free human serum to be used as mock patient samples was filtered and stored at 4 to 8°C; serum to be incorporated into the diluent was adjusted to pH 7.3 to 7.4 before filtering.

Antimicrobial agents. The six antimicrobial agents employed were chosen to be representative of several classes. Stock solutions were prepared from assay powders obtained from the United States Pharmacopeial Convention, Rockville, Md.; final concentrations were 1,280 μg/ml. These stocks were diluted to 1:20 in distilled water and added in equal amounts to pooled human serum to form seeded samples.

MHB. Mueller-Hinton broth (MHB; Difco) was prepared according to the manufacturer's instructions and supplemented with physiologic levels of Mg^{2+} and Ca^{2+} (4). The supplemented MHB was stored at 4 to 8°C and added in equal amounts to heat-inactivated pooled human serum (MHB-S/HS) on each day of testing.

Autobac system. The photometer was operated in the calibrate mode, obtaining light-scattering voltages (LSVs) for each cuvette chamber. Standardization of the bacterial inoculum was performed in the MIC 1 mode with Autobac standardization solution and standardization tubes.

Quality control procedures. The MIC and MBC performance characteristics of the MHB-S/HS were evaluated with a macrodilution test (2.0 ml, final volume). Results were compared with those of Reimer et al. (5); the acceptable range was defined as ±1 tube dilution.

Diluent comparison. Serum inhibitory titers (SITs) and serum bactericidal tests (SBTs) obtained with Autobac low-thymidine eugonic (LTE) broth were compared with macrodilution results obtained in MHB-S/HS (7). The organism-antimicrobial agent combinations tested were *E. coli* 25922-cefazolin and -tetracycline, *Pseudomonas aeruginosa*

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TABLE 1. Bacterial strains and antimicrobial agents examined in clinical isolate studies

Organism (no. of tests)	Antimicrobial agent (no. of tests)
Acinetobacter calcoaceticus (4)	Gentamicin (2), piperacillin (2)
Aeromonas hydrophila (1)	Tetracycline
Alcaligenes odorans (2)	Gentamicin (1), piperacillin (1)
Citrobacter diversus (1)	Tetracycline
C. freundii (4)	Piperacillin (2), tetracycline (2)
Enterobacter aerogenes (5)	Piperacillin (3), tetracycline (2)
E. cloacae (5)	Gentamicin (3), tetracycline (2)
Escherichia coli (10)	Cefazolin (5), tetracycline (5)
Klebsiella oxytoca (2)	Cefazolin (1), tetracycline (1)
K. pneumoniae (8)	Cefazolin (4), tetracycline (4)
Morganella morganii (5)	Cefazolin (3), piperacillin (2)
Proteus mirabilis (5)	Cefazolin (3), Gentamicin (2)
P. vulgaris (5)	Cefazolin (2), piperacillin (3)
Providencia rettgeri (4)	Gentamicin (2), piperacillin (2)
P. stuartii (4)	Gentamicin (2), piperacillin (2)
Pseudomonas aeruginosa (5)	Gentamicin (3), piperacillin (2)
P. cepacia (2)	Gentamicin (1), piperacillin (1)
P. maltophilia (3)	Gentamicin (1), piperacillin (2)
Serratia marcescens (5)	Gentamicin (2), piperacillin (3)
Staphylococcus aureus (20)	Oxacillin (10), penicillin (10)
S. epidermidis (20)	Oxacillin (10), penicillin (10)
Streptococcus faecalis (15)	Penicillin

27853-gentamicin and -piperacillin, *Staphylococcus aureus* 25923-oxacillin and -penicillin. *S. aureus* 29213-oxacillin and -penicillin, and *Streptococcus faecalis* 29212-penicillin. Each series of tests was performed in duplicate to calculate reproducibility percentages. Each organism-antimicrobial agent combination was conducted seven times (63 duplicate trials per diluent = 126 tests per diluent). Doubling dilutions were performed with the diluent and human serum seeded with antimicrobial agents, using a final volume of 1.0 ml per tube.

Organisms were inoculated into 4.0 ml of broth (supplemented MHB or LTE). After 2 to 4 h of incubation at 35°C, the inoculum was adjusted to match the turbidity of a 0.5 McFarland standard and further diluted to 1:20. Colony counts were performed on each diluted inoculum. The adjusted inoculum was added to the tubes with a 100-μl pipettor by inserting the pipette tip beneath the broth surface and flushing to accomplish mixing without agitation of the tubes. In some series, the inoculum was added with a 1.0-ml pipette, and the tubes subsequently were vortexed. Endpoints were compared to evaluate the possibility of an adverse effect of agitation on final SITs and SBTs (1, 8).

Each tube dilution series was incubated for 20 h at 35°C without CO_2, vortexed, and reincubated for 4 h. All tubes were agitated again before recording the SIT. A 10-μl pipette was used to subculture from each visually clear tube to a quadrant of a blood agar plate. After 24 h of incubation, the SBT was recorded as the highest dilution exhibiting growth of five or fewer colonies (≥99.9% death of the original inoculum).

Autobac versus tube dilution. In addition to the organism-antimicrobial agent combinations listed above, three combinations were added: *E. coli* 29194-tetracycline, and *S. epidermidis* 29885-oxacillin and -penicillin. Each combination was tested 10 times (120 tests).

Macrodilution tests were performed with LTE broth and seeded serum samples (2.0 ml, final volume). The inoculum was prepared as previously described and added to each tube with a 1.0-ml pipette. The tubes were then vortexed, and 1.5 ml from each tube was added to a corresponding Autobac cuvette chamber. Chamber 12 received no antibiotic and served as a growth control.

The cuvettes were read immediately on the photometer and were incubated in the incubator-shaker. Further readings were taken at 30-min intervals from 3 to 6 h and again after 20 and 24 h of incubation. The LSVs obtained from these subsequent readings were subtracted from the baseline values to obtain the changes in LSV which had occurred.

The changes in LSV were entered into a computer, and distribution tables were obtained for each organism-antimicrobial agent combination at each of the time periods examined. These tables were used to determine the optimal changes in LSV which could be used as breakpoint values in defining Autobac SIT and SBT results.

The broth remaining in the test tubes (0.5 ml) was incubated at 35°C overnight and examined for inhibitory titers. Subcultures to blood agar were performed to obtain SBTs. These SIT and SBT values were used as a reference for comparing Autobac results.

Standardization comparison. Duplicate macrodilution tests were performed. The inoculum for the series to be transferred to cuvettes was standardized with the Autobac photometer in the MIC 1 mode; the inoculum was diluted to 1:20 before being added to each tube (0.2 ml per tube).

Patient serum samples were prepared by seeding pooled

TABLE 2. Bacterial strains and antimicrobial agents examined in clinical trial studies

Organism (no. of tests)	Antimicrobial agent
Aeromonas hydrophila	Gentamicin
Enterobacter aerogenes	Gentamicin, tobramycin
E. cloacae (2)	Tobramycin
E. coli	Gentamicin
Klebsiella oxytoca	Gentamicin
K. pneumoniae	Cefazolin
Pseudomonas aeruginosa (2)	Tobramycin
Serratia marcescens	Amikacin, tobramycin
Staphylococcus aureus	Penicillin
S. aureus	Tobramycin, vancomycin
S. aureus (2)	Vancomycin
S. epidermidis	Cefazolin
S. epidermidis	Tobramycin
S. epidermidis	Ampicillin, cephalothin, piperacillin
Streptococcus faecalis	Gentamicin, vancomycin

TABLE 3. Correlation results of diluent comparison studies

Organism	Antimicrobial agent	No. of tests[a]			
		SITs		SBTs	
		Similar (±1 dil)	Discrepant (>1 dil)	Similar (±1 dil)	Discrepant (>1 dil)
E. coli 25922	Cefazolinazolin	11	3	14	0
E. coli 25922	Tetracycline	11	3	14	0
P. aeruginosa 27853	Gentamicin	14	0	14	0
P. aeruginosa 27853	Piperacillin	13	1	14	0
S. aureus 25923	Oxacillin	14	0	14	0
S. aureus 25923	Penicillin	9	5	14	0
S. aureus 29213	Oxacillin	12	2	13	1
S. aureus 29213	Penicillin	5	9	14	0
S. faecalis 29212	Penicillin	14	0	14	0
Total (%)		103 (81.7)	23 (18.3)	125 (99.2)	1 (0.8)

[a] dil, Dilution.

human serum with antimicrobial agents. The organism-antimicrobial combinations tested were *E. coli* 25922-cefazolin and -tetracycline, *E. coli* 29194-tetracycline, *P. aeruginosa* 27853-gentamicin and -piperacillin, *S. aureus* 25923-oxacillin, *S. aureus* 29213-penicillin, *S. epidermidis* 29885-oxacillin and -penicillin, and *S. faecalis* 29212-penicillin. Five series of tests were performed with each combination (50 tests). Results were compared with macrodilution endpoints obtained with inocula incubated for 2 to 4 h in LTE and diluted to a 0.5 McFarland standard.

Clinical isolates. Macrodilution tests were performed with inocula standardized with the Autobac photometer. The broth remaining in the test tubes (0.5 ml) after transfer to the cuvettes was incubated and used to obtain tube dilution SITs and SBTs. Autobac results were compared with these values. Seeded serum samples were tested against 135 clinical isolates; the combinations examined are listed in Table 1.

Clinical trials. Patient serum samples were obtained from the Cleveland Clinic Foundation microbiology department and frozen at −20°C until the day of testing. Whenever possible, the infecting organism of the patient was used. If no isolate was available, the serum was challenged with a clinical isolate chosen for its ability to respond to the antimicrobial agent in the sample. The combinations examined are given in Table 2. Test procedures were the same as those described above.

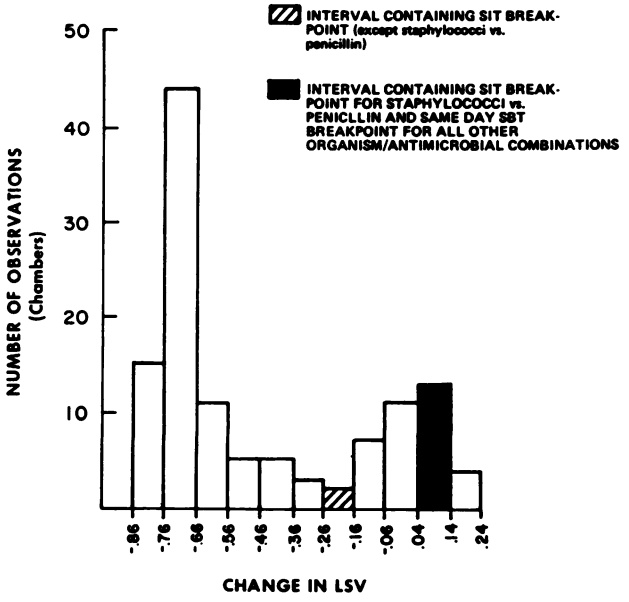

FIG. 1. Representative frequency histogram after 3 h of incubation.

Statistical methods. In the diluent comparison studies, chi-square testing was performed to assess the dependence of SIT and SBT endpoints on the diluent employed. In the remaining experiments, tube dilution results served as reference endpoints. Linear regression analysis was performed by calculating the correlation coefficient (r) from the logarithm of each endpoint dilution.

The acceptable range of comparable results in each series was defined as ±1 tube dilution. Reproducibility percentages were calculated only when each procedure was performed in duplicate on a particular day of testing. The computer programs used to obtain the distribution tables and corresponding histograms were written by T.L.G.

RESULTS

Diluent comparison. The correlation between SITs and SBTs with the two diluents is summarized in Table 3; a chi-square test yielded 33.18 for SIT and 8.96 for SBT. Reproducibility percentages were comparable in both diluents: MHB-S/HS, 98.4 (SITs) and 100% (SBTs); LTE broth, 100% (SITs and SBTs). No significant (>1 dilution) discrepancies in final SIT and SBT results were obtained with either diluent when the inoculum was added with a pipette and the tubes subsequently were vortexed.

Autobac SIT and SBT breakpoints. Immediately following inoculation, the Autobac cuvettes were calibrated on the photometer to obtain a base line of LSVs for each chamber. Subsequent readings were compared with these base-line values. Changes in LSV above the base line were assigned a positive number, while those representing a decrease were assigned a negative number. Since an increase in light scattering causes the voltage reading to fall below the initial value, chambers with substantial bacterial growth would be represented by a large negative number.

The changes in LSV occurring at each of the time periods examined were entered into a computer and were used to obtain distribution tables and frequency histograms for each organism-antimicrobial agent combination. Histograms con-

TABLE 4. Statistical analysis

Study phase	Autobac vs tube dilution	Standardization comparison	Clinical isolates	Clinical trials
% of SITs (±1 dil[a] from reference)	96.7	92.0	90.4	94.4
Correlation coefficient, SITs	0.78	0.78	0.75	0.85
Reproducibility, Autobac SITs	96.7	98.0		
Reproducibility, reference SITs	100	96.0		
% of similar same-day SBTs (±1 dil from reference)	88.9	87.5	83.5	93.8
Correlation coefficient, same-day SBTs	0.75	0.66	0.73	0.89
Reproducibility, same-day SBTs	90.0	90.0		
% of 24-h SBTs (±1 dil from reference)	95.0	98.0	94.8	94.4
Correlation coefficient, 24-h SBTs	0.90	0.93	0.93	0.90
Reproducibility, Autobac 24-h SBTs	98.3	100		
Reproducibility, reference SBTs	100	100		

[a] dil, Dilution.

structed from the distribution tables obtained after 3 h of incubation were examined in detail. A representative example of these histograms is presented in Fig. 1.

Largely through a process of trial and error, an interval was defined which contained the dilution corresponding to the SIT obtained after 24 h with the reference dilution procedure. With the exception of tests involving staphylococci-penicillin, this interval appeared in the same position on the histograms for each organism-antimicrobial agent combination. A separate interval was defined containing the Autobac SIT for staphylococci-penicillin (Fig. 1).

Once these intervals had been defined, medians were calculated and used to obtain Autobac SIT values for comparison with reference procedure endpoints. To eliminate the necessity of having a different breakpoint for each organism-antimicrobial agent combination, each median was used to calculate all Autobac SITs.

The optimal breakpoint values were +0.03 (*S. aureus*-penicillin), +0.15 (*S. epidermidis*-penicillin), and −0.09 (all other organism-antimicrobial agent combinations). These three values were used to compare Autobac and reference procedure SITs in each phase of the study. The results are summarized in Table 4.

This same process was used to obtain Autobac SBT endpoints. With the exception of staphylococci-penicillin, an Autobac endpoint (+0.03) could be defined after 3 h (Fig. 1).

Histograms were also prepared from the frequency distributions obtained after 24 h of incubation (Fig. 2). In this time frame, a breakpoint of −1.00 change in LSV represented a SBT dilution corresponding to the values obtained by subculturing visually clear tubes with the reference method. Table 4 also contains a summary of the comparison between SBT values in each phase of the study.

DISCUSSION

The high degree of disparity between SITs obtained with the reference diluent versus SITs obtained with LTE broth (18.3% of LTE results >1 tube dilution from reference endpoints) may be attributed to the difficulty of reading visual endpoints in a medium containing human serum. This problem has been documented by others (7). However, since SBTs are read by counting colonies on an agar plate, they represent a more objective measurement of comparison. Based on the correlation between bactericidal endpoints in the diluent comparison studies (99.2%), LTE broth was used in devising a procedure for performing the serum bactericidal test with the Autobac system.

Analysis of the frequency histograms obtained in the Autobac versus tube dilution studies led to the definition of changes in LSV which corresponded to SIT and SBT values obtained with the reference tube dilution procedure. For the majority of organism-antimicrobial agent combinations, the optimal SIT value was −0.09; the last cuvette chamber displaying a change in LSV greater than this negative number represents the SIT for the dilution series. In most instances, this value can be obtained after 3 h of incubation with the Autobac system.

Histograms obtained after 3 h with *S. epidermidis* revealed a clustering of data points, indicating poor growth in this time frame. Analysis of the changes in LSV in the growth control chamber revealed that a minimum drop of 0.40 LSV must be achieved before tube dilution SITs could be correlated with those obtained in the cuvette. This change occurred with *S. epidermidis* most frequently after 4 h.

A single value would not correlate cuvette and tube dilution SITs with staphylococci-penicillin. A change of +0.03 was optimal for *S. aureus* (90.0% correlation with tube dilution results), while +0.15 was optimal with *S. epidermidis* (100% correlation).

With the exception of staphylococci tested against penicillin, a same-day SBT breakpoint could be defined with the Autobac procedure. The optimal value was +0.03; the last cuvette chamber displaying a change in LSV >+0.03 represents the SBT for that series of dilutions. For the majority of organisms, the same-day SBT can be determined in 3 h. For both SIT and same-day SBT results, the calculations should

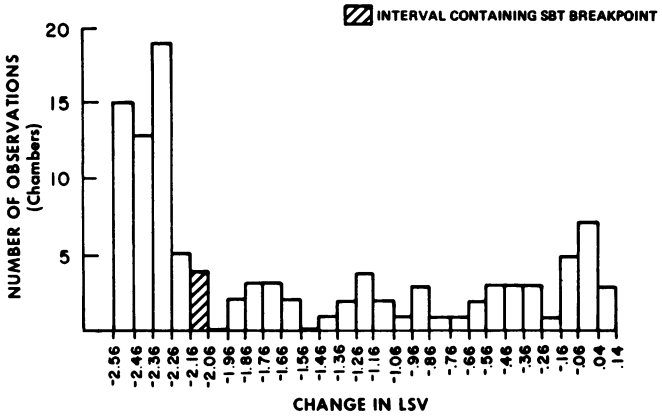

FIG. 2. Representative frequency histogram after 24 h of incubation.

not be performed until there is a minimum drop of 0.40 LSV in the growth control chamber. The overall correlation between Autobac same-day SBTs and reference values calculated after 48 h was 88.4%.

A same-day SBT could not be obtained with staphylococci-penicillin in these studies. However, an extended series of tests utilizing a variety of strains might prove useful in determining this breakpoint. Additionally, studies designed to supplement the LTE broth to provide more rapid growth of staphylococci might result in the determination of a same-day SBT value.

The overall correlation between SBTs calculated after 24 h with the Autobac system and reference values calculated after 48 h was 95.6%. Although the same-day Autobac SBT yielded a somewhat lower correlation (88.4%), this degree of accuracy may still provide some clinical usefulness. In cases of life-threatening infections or in cases of therapy with potentially toxic antimicrobial agents, the same-day SBT may be an important diagnostic aid. The same-day SBT could also be utilized as a preliminary report, followed by a finalized value calculated after the cuvette has been incubated for 24 h.

No attempt was made in these studies to evaluate the effect multiple antimicrobial therapy might have on the Autobac procedure. However, of the 18 samples chosen at random in the clinical isolates study, 5 contained sera with one or more antimicrobial agents (Table 2). With these five samples, there was 100% correlation between Autobac and reference SIT values, 80% correlation between same-day Autobac and reference SBT values, and 80% correlation between 24-h Autobac and reference SBT values (data not shown). While the small number of tests performed precluded any broad conclusions, it would appear that this procedure is not hampered by the presence of more than one antimicrobial agent. However, in cases of staphylococcal infections treated with combination therapy including penicillin, the Autobac procedure could not be used.

In conclusion, the Autobac procedure for performing the serum bactericidal test can provide the clinical laboratory with a reliable alternative methodology which can cut reporting time by as much as 24 h.

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