Influence of Technical Factor Variations on Serum Inhibition and Bactericidal Titors

BERT F. WOOLFREY,* RICHARD T. LALLY, AND KELLY R. TAIT

Section of Clinical Microbiology, Department of Anatomic and Clinical Pathology, St. Paul-Ramsey Medical Center, St. Paul, Minnesota 55101

Received 12 November 1985/Accepted 19 February 1986

The influence of technical factor variations on serum bactericidal and serum inhibitory titers was studied by using Staphylococcus aureus clinical isolates versus oxacillin-spiked human serum. Parallel tests, both with and without the use of β-lactamase in count plates to inactivate oxacillin carryover, were performed with a conventional macrodilution approach, a carefully controlled macrodilution procedure, and a standard microdilution method. Careful control of technical factor variations diminished the incidence of low serum bactericidal titers and decreased the dispersion of results, a finding corollary to the known influence of technical factor variations on the measurement of MBCs. The incorporation of β-lactamase into count plates resulted in a shift of serum bactericidal titers to lower values. The microdilution method appeared to be least influenced by technical variations and, with the addition of β-lactamase to count plates, provided the best results.

Technical factor variations inherent in broth dilution methodology are known to influence significantly the results of MIC and MBC tests. In addition to the need for careful control of media, inoculum preparations, and incubation conditions, it has recently been demonstrated that dilutions should be carefully inoculated below the broth surfaces to avoid spuriously high MBC results, and that care should be taken to prevent antimicrobial-agent carryover from dilution tubes to avoid artifactually low MBC values (5, 12). The technical steps for determining serum inhibition titer (SIT) and serum bactericidal titer (SBT) are similar to those used for determination of MIC and MBC. Although standards for media, inoculum preparation, and incubation conditions have been investigated and proposed (7, 9, 11) for SIT and SBT methodology, the potential production of spuriously low SBT values as a consequence of tube wall contamination and the production of factitiously high SBT values as a consequence of antimicrobial-agent carryover have not been evaluated. The present study investigates the potential influence of these latter two factors on SIT and SBT results.

MATERIALS AND METHODS

Study design. SIT and SBT values were determined for Staphylococcus aureus fresh clinical isolates by three methods performed in parallel: (i) the conventional macrodilution method (macro-A), (ii) a similar macrodilution method that carefully introduces inocula into dilution tubes (macro-B), and (iii) a standard microdilution procedure that uses a single lot of pooled human serum spiked with 128 µg of oxacillin per ml. Parallel tests, without the inactivation of oxacillin, were performed on 30 isolates as both an initial assessment of the control of the inoculation of macrodilutions and a comparison of macrodilution and microdilution results. An additional 26 isolates were then tested by using penicillinase in the colony count plates to assess the effect of preventing oxacillin carryover from the dilution tubes.

S. aureus isolates. Oxacillin-susceptible S. aureus isolates were randomly selected for SIT and SBT testing at the time of isolation in the St. Paul-Ramsey Medical Center Clinical Microbiology Laboratory. Single colonies were selected from primary isolation plates and were streaked to blood agar plates that were incubated at 35°C for 24 h. Using a standard tube dilution procedure (9, 12), we confirmed that MICs for all isolates were below the accepted resistance breakpoint (2 µg/ml) for oxacillin. Correspondingly, as determined by the SIT and SBT testing described below, all isolates were found to have SIT values of ≥1:32.

Macro-A. The conventional serum dilution procedure (7, 11) was performed as follows with a single lot of pooled human serum (Flow Laboratories, Inglewood, Calif.) spiked with 128 µg of oxacillin per ml (Bristol Laboratories, Syracuse, N.Y.). For each isolate, 4 ml of spiked pooled human serum was added to each of the first two tubes in a series of eight sterile glass tubes (16 by 125 mm). An additional 4 ml of pooled human serum with no antimicrobial agent or activity was then added to tubes 2 through 8. Serial dilutions were then performed in tubes 2 through 8 to yield doubling reductions in the oxacillin-spiked pooled human serum concentration from 128 µg/ml in tube 1 to 1 µg/ml in tube 8. A 1-ml volume of each of the oxacillin-spiked serum dilutions from 128 µg/ml through 2 µg/ml was then transferred to a separate series of seven polypropylene tubes (12 by 75 mm) to perform the macrodilution (macro-A) procedure. Tube 8 was retained as a control containing no oxacillin. To each of tubes 1 through 8, 1.0 ml of cation-standardized Mueller-Hinton broth containing 5 × 10^5 CFU of test organism per ml was added by touching the tip of the delivery pipette to the upper surface of the tube and allowing the inoculum to drain down the side of the tube to the surface of the spiked human serum. Tubes were shaken vigorously to mix the contents, and 100 µl was removed from the control tube, diluted 1:100, and plated onto sheep blood agar to serve as a base reference count for SBT determination. All tubes were then incubated for 24 h in air at 35°C. Following incubation, tubes were vigorously vortexed before determination of SIT and preparation of colony count plates. For the 30 isolates tested without addition of penicillinase, 100 µl was removed from each of tubes 1 through 8 and spread confluently onto the surface of separate blood agar plates. For the 26 isolates tested with the addition of penicillinase to the colony count plates, sheep blood agar media was prepared containing 50,000 U of penicillinase.
TABLE 1. Consistency of dilution methods without the addition of β-lactamase to prevent oxacillin carryover

<table>
<thead>
<tr>
<th>Method</th>
<th>No. (%) of isolates with various SBT/SIT ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Macro-A</td>
<td>2 (7)</td>
</tr>
<tr>
<td>Macro-B</td>
<td>4 (13)</td>
</tr>
<tr>
<td>Microdilution</td>
<td>18 (60)</td>
</tr>
</tbody>
</table>

* Of 30 isolates of *S. aureus* challenged by oxacillin and tested by this method, 8 (27%) exhibited the irregular effect and 1 (3%) exhibited the paradoxical effect. Neither effect was exhibited for any of the isolates when tested by either the Macro-B or the microdilution method.

(Penase; Difco Laboratories, Detroit, Mich.) per plate, a quantity sufficient to rapidly inactivate >128 μg of oxacillin per ml (15). For each tube in the SIT series, 100 μl was then removed and plated to separate sheep blood agar plates containing penicillinase. All colony count plates were incubated in air for 24 h at 35°C, and bacterial colonies were enumerated for each plate and compared with the base reference count.

**Macro-B.** The macro-A test described above was modified (macro-B) so that serum dilutions could be inoculated carefully and incubated along the lines suggested by Ishida and associates (5) and Taylor and associates (12) in their investigations of broth macrodilution MIC and MBC testing. One ml of each of the oxacillin-spiked pooled human serum dilutions prepared as described above in the tubes (16 by 125 mm) was diluted with 1 ml of cation-standardized Mueller-Hinton broth and was then transferred to a separate series of seven polypropylene tubes (12 by 75 mm) to produce an oxacillin dilution series from 64 μg/ml in tube 1 to 1 μg/ml in tube 7. Tube 8 was retained as a control containing no oxacillin. To each of tubes 1 through 8, 100 μl of cation-standardized Mueller-Hinton broth containing 5 × 10^6 CFU of test organism per ml was added by using a micropipette. The inoculum was delivered below the surface of the pooled human serum and gently refluxed to avoid bubbling or splashing of contents onto the tube walls. Tubes were incubated for 24 h in air at 35°C with no additional agitation. Following the incubation period, all tubes were vigorously vortexed and reincubated for an additional 4 h. SIT determinations were then made, and colony count plates were prepared as described above for macro-A.

**Microdilution test.** A microdilution procedure patterned after the recommendations of Shanholzer and associates (10) was performed as follows. For each test isolate, 96-well microtiter panels (Dynatech Laboratories, Inc., Alexandria, Va.) were divided to provide separate 8-well series. From the original tubes (16 by 125 mm) containing oxacillin-spiked pooled human serum dilutions, 50 μl was delivered to each of the eight wells in the test series, providing concentrations analogous to those in macro-A. To each well in the series, 50 μl of cation-standardized Mueller-Hinton broth containing 5 × 10^6 CFU of test organism per ml were added with a micropipette and gently refluxed to mix. Panels were incubated for 24 h in air at 35°C, at which time SIT determinations were made and the entire contents (100 μl) of each well was removed for subculture to count plates as described for the macrodilution procedures. For each of the 26 isolates that were used to investigate the influence of adding penicillinase to count plates, two parallel microdilution tests were performed and were subcultured separately to count plates with and without penicillinase.

**SIT and SBT determinations.** SIT was defined as the highest titer of oxacillin-spiked serum for which no visible growth was evident after 18 to 24 h of incubation. SBT was defined as the highest titer of oxacillin-spiked serum for which the subculture plate count was at least 2 standard deviations below the reference count, indicating 0.1% survival. The irregular effect was defined as the occurrence of one or more consecutive, erratic plate counts greater than the 0.1% reference count, but with serum titers lower than the SBT. When the irregular effect occurred, SBT was defined as that serum dilution titer for and below which plate counts were consecutively <0.1% of the reference count. The paradoxical effect was defined as the occurrence of three or more consecutively increasing plate counts, showing titers equal to or less than the SIT. When the paradoxical effect was observed with some or all of the peak counts exceeding 0.1% of the original inoculum density, SBT was defined as that serum titer for and below which plate counts were consecutively <0.1% of the original inoculum density. The SBT/SIT ratio was defined as the serum titer representing the SBT divided by the serum titer representing the SIT for each method.

**RESULTS**

Table 1 shows the distribution of SBT/SIT ratios for 30 oxacillin-susceptible *S. aureus* clinical isolates in parallel macro-A, macro-B, and microdilution tests without β-lactamase added to count plates. Results for the less well controlled macro-A procedure show a moderate but distinct predominance of higher SBT/SIT ratios as compared with those obtained by the carefully controlled macro-B method. Additionally, SBT/SIT ratios for the microdilution method were distinctly lower and less dispersed than the macrodilution results. For the macro-A procedure, 27% of SBT interpretations were complicated by the occurrence of the irregular effect, a situation not encountered for either macro-B or microdilution.

Table 2 presents the frequency distributions for SBT/SIT results, using parallel macro-A, macro-B, and microdilution tests and adding β-lactamase to count plates. The macrodilution SBT/SIT ratios were again somewhat larger and more dispersed for the macro-A test than for the carefully controlled macro-B procedure. Both macro-A and macro-B results with penicillinase were slightly larger and more dispersed than those summarized in Table 1 for the same tests without penicillinase. Tables 1 and 2 show that incorporation of penicillinase into the microdilution procedure produced a

**TABLE 2. Consistency of dilution methods with the addition of β-lactamase to prevent oxacillin carryover**

<table>
<thead>
<tr>
<th>Method</th>
<th>No. (%) of isolates with various SIT/SBT ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Macro-A</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Macro-B</td>
<td>4 (15)</td>
</tr>
<tr>
<td>Microdilution</td>
<td>11 (42)</td>
</tr>
<tr>
<td>Microdilution without β-lactamase</td>
<td>17 (65)</td>
</tr>
</tbody>
</table>

* Of 26 isolates of *S. aureus* challenged by oxacillin and tested by this method, 12 (46%) exhibited the irregular effect, and 0 exhibited the paradoxical effect.
* Of the 26 isolates tested by this method, 7 (27%) exhibited the irregular effect, and 0 exhibited the paradoxical effect.
* Of the 26 isolates tested by this method, 3 (11%) exhibited the irregular effect, and 1 (4%) exhibited the paradoxical effect.
* Of the 26 isolates tested by this method, 0 exhibited either the irregular or the paradoxical effect.
definite but small shift towards higher and more disperse SBT/SIT ratios. The irregular effect was notably increased for all three procedures when β-lactamase was used, a factor that was particularly evident for macro-B.

DISCUSSION

In 1947, Schlichter and MacLean (8) proposed SIT as a useful means of monitoring antimicrobial therapy. Five years later, Fisher (4) extended the test to include SBT. Since then, using many methodologic variations, millions of such tests have been performed in both investigative and clinical applications. In recent comprehensive reviews, Coleman and associates (3) and Wolfson and Swartz (14) have found little evidence to support the clinical usefulness of SIT and SBT testing and have stressed the need for future methodologic standardization and well-designed clinical trials for proper evaluation of what should be in theory a useful procedure. Coincident with the Wolfson and Swartz review, the results of a large collaborative study (13) were published, indicating that therapeutic success for treatment of endocarditis could be correlated with SBT peak values of ≥1:64 and trough values of ≥1:32, although lower values could not be correlated with either success or failure. Several centers were involved in the study, and SBT values were determined by a reportedly standardized microdilution test. The test was performed without β-lactamase incorporation and used a combination of human serum and Mueller-Hinton broth as the diluent. The latter had been shown (6, 7, 11) to be an acceptable medium, with buffering capacity similar to that of Mueller-Hinton agar and protein-binding capacity close to that of serum.

Because of analogous methodologies, SBT measurements can suffer from dependency on the same technical factor variations that have been shown to markedly affect MBC results. It has been demonstrated (5, 12) that spuriously high oxacillin MBCs are frequently produced for S. aureus isolates through the conventional macrodilution method. In contrast, spurious results are essentially eliminated when the following principles are incorporated into the technique: (i) use of log-phase inoculum, (ii) careful introduction of inoculum below the dilution broth surface, (iii) thorough mixing of dilution cultures after 20 h of incubation, (iv) thorough mixing prior to the time of subculture into count plates after 24 h, and (v) addition of β-lactamase to count plates to prevent oxacillin carryover. Studies of microdilution applications for MBC testing have also demonstrated significant technical factor dependency (1, 2, 10, 16), the effects of which can largely be eliminated by careful control of the same factors that influence macrodilution results. Despite the similarity of SBT and MBC methodologies, investigations concerning the influence of technical factor variations on SBT results have not yet been reported.

Our findings indicate that spuriously low SBT values occur more frequently and results are more disperse when technical factors are not carefully controlled for the macrodilution method. Spuriously low values were less frequently encountered and dispersion was less for microdilution than for other methods, suggesting that technical factor variations are inherently easier to control or perhaps are less significant for the microdilution approach. As compared with MBC results using similar methodologies (5, 12, 15), both macrodilution and microdilution SBT results appear markedly less influenced by technical variations. This unexpected finding remains unexplained but could be related to the use of human serum in the SBT diluent mixture, in contrast to the conventional use of Mueller-Hinton broth for MBC tests. For both macrodilution and microdilution procedures, we found higher and less disperse SBT results when β-lactamase was not used.

A recent collaborative study on the therapeutic significance of high SBT values used a microdilution test, apparently similar to that used in the present study, although the exact technical details were not completely outlined in that report (13). Our findings suggested that technical factor variations do not markedly influence microdilution SBT values; however, the tests done by Weinstein and associates were performed at several laboratories, suggesting the possibility of suboptimal control of technical variations. This factor may have resulted in an increased frequency of low values, a situation that could account for the failure to correlate low titers with therapeutic failure. Conversely, and compatible with our findings, failure to incorporate β-lactamase into the test procedure may have shifted test results toward higher values, a situation that might account for the correlation of therapeutic success with somewhat higher peak and trough titers than those previously suggested by other investigations.

With careful control of methodologic variables and the addition of an appropriate β-lactamase to count plates, we demonstrated that reliable SBT values can be obtained by both the macrodilution and microdilution approaches, the latter being preferred on the basis of both reliability and technical simplicity. For patients on other than β-lactam therapy, the inability to incorporate inactivating agents onto count plates should provoke the suspicion that SBT values may be artifactually high. The interpretation of results now remains the major problem. Subjectively attractive and difficult to argue against is the use of SBT to monitor successful conversion from intravenous therapy to oral therapy. For a specific disease process, results from the study by Weinstein and colleagues on endocarditis appear to provide the best guidelines to date (13), but their results need confirmation and an improved interpretation of low and midrange values. For other infections, interpretive guidelines remain unestablished, and well-designed clinical investigations using carefully controlled methods are greatly needed.

ACKNOWLEDGMENT

This research was supported by grant 8-381 from the St. Paul-Ramsey Hospital Medical Education and Research Foundation.

LITERATURE CITED
6. Pien, F. D., R. D. Williams, and K. L. Vosti. 1975. Comparison of broth and human serum as the diluent in the serum bacteri-


