Technical Aspects of the *Staphylococcus aureus* Teichoic Acid Antibody Assay: Gel Diffusion and Counterimmunoelectrophoretic Assays, Antigen Preparation, Antigen Selection, Concentration Effects, and Cross-Reactions with Other Organisms

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Because variable results are being reported from laboratories performing the teichoic acid antibody assay in patients with serious infections due to *Staphylococcus aureus*, we have thoroughly reviewed all technical aspects of the test. This paper reports on the importance of agar and antigen preparation in standardizing results of the assay and reducing the prevalence of false-negative and -positive tests. Once standardized, the counterimmunoelectrophoretic method is as accurate as the gel diffusion method for both initial screening and generating titers; practically, however, unless numerous tests are to be performed, the gel diffusion technique will suffice for most clinical purposes. The cell wall of the Lafferty strain of *S. aureus* was used as the standard antigen in assays for serum antibodies. We studied whether it is an appropriate antigen and found that antibody titers obtained with the Lafferty strain antigen were, in three patients with endocarditis, the same as those obtained with antigens from the individual blood stream isolates. We have also confirmed that pooled human gamma globulin can be used, by back titration against newly prepared lots of antigen, to select optimal antigen concentration and is as good as more specific, higher titer serum specimens for that purpose. Finally, cell wall antigens from *Staphylococcus epidermidis* and a variety of streptococci may react with normal human sera, but such antigens are distinct by immunoprecipitation from those from *S. aureus*.

Rising or elevated serum levels of antibodies to staphylococcal cell wall teichoic acid correlate with the development of serious infections due to *Staphylococcus aureus* (2, 7, 10–12). However, levels of teichoic acid antibodies have varied substantially both in normal control populations and in patients with various types of staphylococcal infections. Using *S. aureus* endocarditis as an example, Crowder and White (2), in their initial description of usefulness of the gel diffusion assay for the detection of antibodies to teichoic acid, found that 14 of 15 patients with endocarditis due to *S. aureus* had measurable antibody levels; none of 22 patients with endocarditis caused by other organisms had such antibodies. In a series of 64 drug-abusing patients with right-sided endocarditis, we found that 91% were positive by the gel diffusion assay, whereas 98% gave positive reactions by the counterimmunoelectrophoresis (CIE) assay (10). The latter assay was clearly more sensitive but was less specific in that up to 20% of healthy drug users were found to have measurable antibodies. Recently, Wheat et al. (12), using a radioimmunoassay specific for immunoglobulin G antibodies to teichoic acid, found that only 49 of 58 (84%) patients with endocarditis or "complicated" bacteremia were positive. Thus, as many as 10 to 15% of patients with clear-cut endocarditis due to *S. aureus* may not develop or show a rise in preexisting antibodies to teichoic acid.

A recent *Lancet* editorial (4) has pointed out that whereas teichoic acid antibody tests are quickly and easily done, they seem to be especially unreliable in identifying the patients with chronic, deep-seated infections such as osteomyelitis.

Thus, it has been difficult to interpret results of reported antibody titers and to draw conclusions as to the usefulness of the assay. Much apparent variability between laboratories is likely to be due to differences in technique. We, therefore, are reporting on several technical aspects of the teichoic acid antibody assay such as
the method of antigen preparation and concentration, choice of antigen to use in the assay, possible cross-reactions with other organisms, the type of gel to use in the immunodiffusion assay, and a comparison of titers obtained using CIE as opposed to passive diffusion in agar gel.

MATERIALS AND METHODS

Gel diffusion assay. The method of performing the gel diffusion assay has been described in detail previously (2, 6, 7). Briefly, laboratory-prepared plates were made by using petri dishes (100 by 15 mm) and 1.2% agarose in a phosphate buffer (0.05 M, pH 8.0). Antigen was usually placed in the center well and antisera were placed in the outer wells. All wells were 4 mm in diameter, and the center-to-center interwell distance was 8 mm. For part of these studies, we also used commercially purchased plates (Meloy Laboratories, Springfield, Va.). Both types of plates were incubated at room temperature in a humidity chamber and examined the next day (at approximately 18 h), using a Pfizer Diagnostic View Box (Pfizer Laboratories, Diagnostic Division, New York, N.Y.). To properly recognize a positive test result, one must read the proper precipitin line. As a standard, known positive antiserum, we use pooled, concentrated human gamma globulin (HGG) (Gammagee, obtained from Merck Sharp & Dohme, West Point, Pa.), which reproducibly gives the proper teichoic acid line in agar gel up to a 1:8 dilution. A large supply of this material was purchased and pooled so that a single preparation was (and will be) available for use as a standard antiserum. To define what line is the proper one, the pooled HGG preparation must be reacted against purified ribitol teichoic acid (RTA). We prepared the purified antigen in our lab by the method used by Peterson et al. (8). We also obtained purified RTA from G. W. Ross (The Glazo Laboratory, Greenford, England). Our preparation and the one from Dr. Ross were identical, and purified RTA is precipitated in a line of identity with the heaviest precipitin line produced by the interaction of the whole Lafferty strain antigen and HGG (Fig. 1). Figure 2 illustrates where the proper line is located on the usual type of agar plate. We suspect that this line is due primarily to beta-RTA. Another line, always nearer the serum-containing well, is present in some sera (see Fig. 2), but we believe it is not the line to be read diagnostically. We presently suspect that this outer line is due to alpha-RTA and are presently evaluating whether or not the presence of that line has any diagnostic significance. Other precipitin lines may occasionally be seen inside these lines, the most common of which are apparently protein A lines (2, 3; see Fig. 3). These apparent protein A lines are eliminated by pretreating the antigen preparation with trypsin (see below), thus reducing to a minimum any confusion between these sometimes prominent inner lines and the true teichoic acid precipitin line. Further studies are in progress to define which cell wall antigens produce these and other lines sometimes seen in clinical specimens.

Significant differences were found in antibody titers determined on the agar plates made in our own laboratory when compared with those purchased commercially. Our own plates consistently yielded a one- to twofold-higher titer than the commercial plates (Table 1). After recognizing this fact, we thereafter used only plates which we prepared in generating further data. The Meloy immunodiffusion plates were hazier, and the lines which developed were more diffuse. Thus, the highest positive dilution was more difficult to read precisely.
TABLE 1. Comparison of teichoic acid antibody titers determined on gel diffusion plates prepared in our laboratory versus those read on plates purchased from a commercial laboratory

<table>
<thead>
<tr>
<th>Titer read on purchased plate</th>
<th>No. of serum specimens tested with given titer read on VA laboratory-prepared plates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Negative</td>
<td>16</td>
</tr>
<tr>
<td>Undiluted</td>
<td>0</td>
</tr>
<tr>
<td>1:2</td>
<td>0</td>
</tr>
<tr>
<td>1:4</td>
<td>0</td>
</tr>
<tr>
<td>1:8</td>
<td>0</td>
</tr>
<tr>
<td>1:16</td>
<td>0</td>
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</tbody>
</table>

Fig. 3. Precipitin lines in agar gel between a whole, untrypsinized antigen (DA) made from a patient’s organism and serial dilutions of the standard pooled HGG. (See legend to Fig. 2 for explanation of labels.) The heavy outer line is the teichoic acid-antibody reaction; the linear lines are nonspecific reactions between protein A and antibodies. Such inner lines are eliminated by trypsinization of the antigen preparation.

CIE assay. CIE determinations were carried out on glass slides (4 by 5 in. [ca. 10 by 12 cm]) covered with 1.2% Noble agar (Difco Laboratories, Detroit, Mich.) in barbital buffer (0.025 M, pH 8.6). It was found that precoating the slide with 0.1% Noble agar produced better adhesion. Antigen and antibody wells, 4 mm in diameter, were punched in the agar with a center-to-center interval distance of 10 mm. Up to 18 sets of antigen-serum combinations can be run on a single slide (Fig. 4). Electrophoresis was performed with a barbital buffer solution (0.05 M, pH 8.6) for 40 min at a constant voltage of 250 V across the prepared plates. Antigen was placed in the cathodal wells, and twofold dilutions of test sera were placed in the anodal wells. A known positive control serum from a patient with endocarditis is run on each plate to assure consistent results. Of interest, and as yet unexplained, is the observation that the use of the gamma globulin control does not give a precipitin line in the CIE assay. Thus, we run a known positive, high-titer patient’s serum as a positive control on each CIE plate. The plates were washed in saline after the CIE run for approximately 10 min to remove nonspecific proteins and then read at once, using a diagnostic view box as noted above. The formation of a clear-cut precipitin line between the antigen and antibody wells was considered a positive result (Fig. 4).

Antigens. The Lafferty strain of S. aureus (phage type 80-81) was obtained from Arthur White (University of Indiana School of Medicine, Indianapolis). Studies have shown that this strain is quite appropriate to use for screening for teichoic acid antibodies in patients’ sera (2); it contains sufficient amounts of both alpha- and beta-RTAs to detect both types of antibodies (A. White, personal communication). The method of antigen preparation from the Lafferty strain is precisely outlined below. Organisms from patients with endocarditis were obtained from blood cultures: the “Te” strain culture was obtained from Alan Miles (St. John’s Hospital, Detroit, Mich.). The “Ca” and “Da” strains were from patients hospitalized at the Ann Arbor Veterans Administration Medical Center. Four strains of Staphylococcus epidermidis were available from patients with endocarditis. Also, four strains of streptococci (three penicillin-sensitive alpha-hemolytic strains and one enterococcus strain) from cases of endocarditis were similarly available. Also studied was a strain of group B and one of group G streptococcus obtained from the American Type Culture Collection (Rockville, Md.). Antigens prepared from these organisms were studied in both CIE and agar gel diffusion against sera from patients with S. aureus endocarditis to ascertain cross-reactivity. Similarly, sera from the eight patients with endocarditis (due to S. epidermidis and the streptococci) were available for testing against the Lafferty strain antigen.

Antigen preparation. A 12-ml amount of brain heart infusion agar was placed in each of five petri dishes (100 by 15 mm). Each plate was heavily inoculated with the organism to be studied. The organism was grown for 40 to 48 h on the brain heart infusion agar plate at 37°C and then harvested by suspending the organism on the plate in sterile 0.9% NaCl (9 ml per dish), using a rubber policeman. The suspended organisms were then decanted into 50-ml centrifuge tubes and centrifuged for 30 min at 5,000 rpm. The organisms were resuspended in 0.9% NaCl and were then killed by adding 1 ml of 0.1% sodium azide per 50 ml of suspension. The organisms were again centrifuged, the supernatant was removed and discarded, and the packed bacteria were washed three times with 0.9% NaCl. After the final centrifugation and wash, the supernatant was again discarded and the packed organisms were resuspended in 0.9% NaCl to a total of 10 ml. The suspended organisms were then homogenized in a Braun homogenizer (Braun Instrument Co., Berkeley, Calif.) for 30 min for staphylococci and 5 min for streptococci. This procedure resulted in disruption of over 95% of the suspended organisms as assessed by Gram stain. A 0.1-mg portion of trypsin...
was added, and the mixture was incubated for 30 min. The raw suspension was then boiled for 30 min to inactivate any autolysins as well as any residual trypsin and then centrifuged at 10,000 rpm for 10 min. The supernatant was saved and dialyzed against 2.5 liters of distilled water for 2 days. This approximately 10-ml portion of dialyzed supernatant was considered "concentrated antigen" and contained from 2 to 15 mg (dry weight) of the cell wall components per ml.

The concentration of a given antigen to be used in the assay was determined by serially diluting the antigen and titrating each antigen dilution against HGG. The proper antigen concentration for the assay was chosen as that which produced the clearest line at the highest titer, using HGG. Antigens produced from patient isolates (Te, Da, and Ca) were titrated for comparison against both HGG and the patient's own serum. As almost all normal persons have moderate to high titers of antibodies against the antigens prepared from the S. epidermidis and streptococcal strains, back titration to define an appropriate antigen concentration was carried out against a single large sample of serum from one of the authors (J.N.S.). All antigens were stored at 4°C until used.

Lysostaphin preparation of antigen. Lysostaphin preparation of antigen was done only with the Lafferty strain organism. Organisms were handled identically to preparation of antigen with the Braun homogenizer up to the point of homogenization. At the point where the washed organisms were resuspended in four times their volume of 0.9% saline, lysostaphin (Sigma Chemical Co., St. Louis, Mo.) was added at a concentration of 10 U/ml of total suspension. The mixture was then incubated at 37°C on a rotator for 2 h, boiled for 30 min, and centrifuged for 10 min at 10,000 rpm. The supernatant was saved, combined with the supernatants of two additional 10- to 15-ml saline washes, and dialyzed against distilled water for 2 days. This material is considered to be the concentrated lysostaphin-prepared antigen. The lysostaphin-prepared antigen was used at a concentration of 0.5 mg/ml for CIE because that concentration seemed to give maximum visualization of precipitin lines. For the gel diffusion assay, a concentration of 1.0 mg of the antigen per ml was used; again, this concentration maximized titers in known positive antisera in pretest trials.

Patients' sera. Serum samples to be tested against each antigen were obtained from patients suspected of having a variety of serious infections with S. aureus (usually endocarditis, osteomyelitis, or a complicated bacteremia). Each antigen was also tested against several samples of serum from normal, uninfected individuals. All serum samples were stored frozen at -20°C and were then thawed and stored at 4°C during the course of these experiments. The control antiserum (tested along with each serum sample) was, as noted above, HGG.

**RESULTS**

**Influence of different methods of antigen preparation.** Initially, and as yet unexplained, a large number of "false negative" sera by CIE were found with the lysostaphin-prepared antigen: i.e., many patients were positive by the gel diffusion technique but negative by CIE. Thus, it seemed that the CIE method might not be as useful as hoped as a more rapid screening test for the presence of teichoic acid antibodies. Because of the increased sensitivity of CIE over gel...
TEICHOIC ACID ANTIBODY ASSAY

TABLE 2. Comparison of teichoic acid antibody titers determined by the CIE technique as opposed to the gel diffusion technique

<table>
<thead>
<tr>
<th>Titer determined by</th>
<th>No. of serum specimens tested with given titer determined by gel diffusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CIE</strong></td>
<td><strong>Negative</strong></td>
</tr>
<tr>
<td>Negative</td>
<td>20</td>
</tr>
<tr>
<td>Undiluted</td>
<td>4</td>
</tr>
<tr>
<td>1:2</td>
<td>6</td>
</tr>
<tr>
<td>1:4</td>
<td>0</td>
</tr>
<tr>
<td>1:8</td>
<td>0</td>
</tr>
<tr>
<td>1:16</td>
<td>0</td>
</tr>
<tr>
<td>1:32</td>
<td>0</td>
</tr>
</tbody>
</table>

* Of this group of four samples, three of the four had lines which were not identical to the beta-RTA line and are not therefore true positives.

diffusion, many false-positive tests are expected (i.e., positive by CIE but negative by gel diffusion), but false-negative results would severely limit the usefulness of the CIE method. The homogenized antigen preparation, however, essentially eliminated this problem. Only four false-negative titers (i.e., negative by CIE but positive by gel diffusion) were found of 120 serum specimens tested (Table 2). Three of these four samples had lines in gel diffusion which were not identical to the beta-ribitol line and therefore were not true positives. It is not surprising that the CIE method picks up antigen-antibody reactions other than the one between beta-RTA and antibody. The homogenized antigen was clearly the best one to use in both the CIE and gel diffusion assays.

Relationship between antibody titers obtained by CIE and those obtained by gel diffusion. The homogenized antigen was then used to explore the precise relationship between titers determined independently by the CIE and gel diffusion techniques under controlled conditions. As expected, the correlation between the titer defined by the gel diffusion assay and that defined by the CIE method was excellent (see Table 2). CIE was generally more sensitive than gel diffusion, especially in patients with titers of >1:4. We continue to define “true” antibody titers by the gel diffusion assay after initial screening by CIE. With our methods, 80% of 250 normal controls have no detectable antibodies by gel diffusion; however, 7% are positive in undiluted serum specimens, and 3% are positive at a 1:2 dilution. Thus, at present, in our laboratory a titer by gel diffusion of 1:2 or greater is highly suggestive of an ongoing, or recent, serious disseminated infection with *S. aureus*. Such titers continue to be observed in over 90% of patients with endocarditis due to *S. aureus* by the end of week 2 of clinical illness (10).

Selection of proper antigen concentration. The relationship between the concentration of antigen and the titer of HGG is shown in Fig. 5. Varying antigen concentration appreciably affects the titer of antibody in the gel diffusion assay. Both peak HGG titer obtainable and clarity of the line produced are important in deciding on the optimal antigen concentration. The relationship of antigen concentration (in milligrams per milliliter) to titer of HGG varies noticeably with the strain of organism used and even with the same strain prepared at a different time. Therefore, the concentration of antigen which gives the highest titer against HGG must be determined separately each time a new antigen is prepared. In fact, the amount of “antigen” by dry weight of a given antigen preparation was so variable that simple titration of the final concentrated antigen in fluid phase turned out to be the best method of standardization. Using this technique, previously determined antibody titers in stored sera could be reliably reproduced. The optimal concentration of all antigens identified by back titration against the patient’s own serum was always within one tube dilution of that obtained with HGG. Thus, titration against HGG is a satisfactory method of standardizing newly prepared lots of any staphylococcal antigen.

Lafferty strain versus the patient’s own organism as antigen. Antibody titers in serum samples from three patients (samples Te, Da, and Ca) tested against antigens prepared from the patient’s own infecting organisms as well as against the Lafferty strain antigen are shown in Table 3. The Lafferty strain antigen always gave a titer in a given specimen of serum the same as or within one dilution of that obtained with the patient’s own organism as the antigen. Interestingly, using other patient’s infecting organisms to determine antibody titers in a given serum specimen resulted in titers essentially identical to those obtained with the Lafferty strain antigen; in only one case was the titer consistently lower (Ca serum titrated with the Te organism; see Table 3).

Cross-reactions between antigens from the Lafferty strain and other organisms. The pooled HGG standard antiserum on occasion exhibits a faint line against each of the antigens made from the four strains of *S. epidermidis* studied. The reason for this variability is unclear but is probably due to day-to-day technical differences when the antibodies are just at the level of detectability. However, as stated previously, individual patient and control sera regularly contain high titers (1:4 to 1:64) of
antibodies against cell wall antigens made from all of the *S. epidermidis* strains. Why individual sera should have high titers while the pooled immunoglobulin G does not is totally unclear. Such precipitin lines are easily differentiated from the two lines detected with the *S. aureus* Lafferty strain antigens (Fig. 6), and no cross-reactions were seen to occur. Interestingly, the titer of these naturally occurring antibodies against *S. epidermidis* antigens does not seem to change over the course of an episode of endocarditis even when caused by the organism from which the antigen was obtained. Further studies are under way to verify this observation.

Antigens were also made from the six strains of streptococci described earlier and were reacted against pooled HGG; two of the four strains from infected patients (the enterococcus strain and one strain of viridans streptococcus) showed no reaction. However, as occurred with the *S. epidermidis* strains, antigens from all six streptococcal strains produced clear precipitin lines in high titers when reacted against a variety of individual sera from patients infected with both staphylococci and streptococci, as well as against those from normal persons. The precipitin lines detected with each of these streptococcal antigens are identical among themselves but are not identical with either of the definable *S. aureus* antigens. Sera from patients with these infections never produced visibly identifiable precipitating antibodies when reacted against the Lafferty strain antigen; thus, in these cases, whether evaluating the antibodies in pooled HGG or in individual sera, no cross-reacting antibodies against *S. aureus* teichoic acids were demonstrable.

**DISCUSSION**

Careful attention to the technical aspects of preparation of both agar and antigen is critically important to the quality of both the CIE and gel diffusion assays for teichoic acid antibodies. Comparison between gel diffusion plates prepared in our own laboratory and those purchased commercially show agar preparation to be a definite determinant of the sensitivity of the gel diffusion assay.

As an antigen, the Lafferty strain of *S. aureus* continues to function quite satisfactorily. However, the method of antigen preparation also proves to be important. The sonication method

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**TABLE 3. Reproducibility of teichoic acid antibody titers, using either the Lafferty strain antigen or an antigen from the patient's own infecting organism.**

<table>
<thead>
<tr>
<th>Serum specimen (data obtained)</th>
<th>Antibody titer using antigena</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>La</td>
</tr>
<tr>
<td>Pooled HGG</td>
<td>1:8</td>
</tr>
<tr>
<td>Te (5/24/78)</td>
<td>1:8</td>
</tr>
<tr>
<td>Da (4/11/78)</td>
<td>1:4</td>
</tr>
<tr>
<td>Ca (2/3/78)</td>
<td>1:16</td>
</tr>
</tbody>
</table>

*a* Antigens: La, Lafferty strain of *S. aureus*; Te, patient Te's own organism; Da, patient Da's own organism; Ca, patient Ca's own organism. All antigens were used in the optimal concentration identified by back titration against pooled HGG (see Fig. 4).
(using a Braun sonicator) clearly produced fewer false-negative results than the lysostaphin method. Antigen preparation may well play a role in the generation of the variable clinical test results obtained by other laboratories (4, 12).

The CIE method is highly sensitive in detecting the presence of the same teichoic acid antibodies as does gel diffusion (7) and is clearly useful as a screening test. It is of interest, however, that the CIE assay is occasionally negative when the gel diffusion assay seems to be positive; in these cases, the lines seen in agar gel are not those against beta-RTA and should at this time not be considered false-negative tests. Thus, for a busy laboratory screening large numbers of serum samples, the CIE technique is quite suitable as a method of both screening and titrating antibody levels for labs so equipped; however, it is technically more cumbersome than the gel diffusion method. The only advantage of the CIE assay is that, when required, the results of the titration are available in 30 to 45 min as opposed to overnight for the gel diffusion assay. Gel diffusion titration will clearly suffice for most laboratories where neither large-scale assaying nor the immediate availability of titration data is required. Also, visual identification of which precipitin line is present is possible with the gel diffusion assay. As stated previously, and for unknown reasons, the CIE technique cannot utilize the commercial gamma globulin preparation as a known positive serum; no line appears on the CIE slide.

Different results reported by various investigators may be explained by lack of standardization of the techniques involved in the performance of the teichoic acid antibody assay. However, precise methodology for this assay based on a thorough understanding of all variables involved results in reproducible data. Clearly, a commercially available, standardized assay system needs to be developed.

For freshly prepared antigens, the optimal concentration can be identified by titration of various antigen concentrations against a pooled HGG standard. The HGG standard results in the selection of the same optimal antigen concentration as that selected with immune patient sera for either the Lafferty strain of *S. aureus* or the patient's own organism. Thus, HGG can reliably be used for antigen standardization.

We have verified that the Lafferty strain antigen is an adequate screening antigen to measure antibody titers in clinical specimens. We compared the titers obtained on three different patient serum specimens, using the Lafferty antigen with titers obtained in the same serum specimens with an antigen prepared from the organism isolated from the patient's bloodstream. That is, the antibody titer present in a given patient serum specimen was found to be essentially the same with either the Lafferty strain antigen or an antigen prepared from the patient's own organism. Also, with only one exception (the Ca titer, using our antigen from the Te organism), the titers obtained with antigens from individual patient organisms were essentially the same as those obtained with the Lafferty strain antigen. In nature, we have found that pathogenic strains of *S. aureus* contain predominantly beta-RTA residues in fairly constant amounts (6). The Lafferty strain of *S.
aureus, fortunately, also contains predominantly beta residues and thus is a satisfactory screening antigen for clinical purposes.

Finally, some faint lines are occasionally detectable with S. epidermidis or streptococcal antigens against the HGG standard and are regularly detected in moderate to high titer in serum from normal control subjects as well as from patients with S. aureus infections; however, such lines are not identical to S. aureus precipitin lines. Conversely, none of the eight serum specimens from the patients suffering either S. epidermidis or streptococcal endocarditis showed antibodies against S. aureus antigens. This was a small sample of such patients. Previous publications have shown that cross-reactions with antigens from S. aureus may occur in some patients with S. epidermidis (2), streptococcal (6), or diphtheroid (5) endocarditis. Also, antigens in the cell wall of Haemophilus influenzae (type 3) have been observed to cross-react with S. aureus antigens both in vitro (1) and in vivo (9).

It is hoped that further investigations of the technical aspects of this assay will improve its value as a diagnostic tool. There is clearly a need for serological tests which, alone or in combination, can help either to identify a high proportion of patients with deep staphylococcal infections or, possibly more importantly, to rule out such complications in bacteremic patients (11).

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LITERATURE CITED