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# Anticoagulant Carryover May Influence Clot Formation in Direct Tube Coagulase Tests from Blood Cultures

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**The tube coagulase test (TCT) performed directly from positive blood culture bottles has been used to reduce the turnaround time for identifying *Staphylococcus aureus*. Most reports have shown the test to be specific but often lacking sufficient sensitivity to be useful. In a prospective study of blood culture bottles (BCB) signaling positive, with a Gram-stained smear showing gram-positive cocci resembling staphylococci, the sensitivity of the direct TCT was improved by diluting the BCB broth 1:10 in saline before inoculating 0.1 ml into 1.0 ml of 10% pooled human plasma. It was hypothesized that the improved sensitivity might be explained by reduced carryover of the anticoagulant sodium polyanetholesulfonate (SPS) used in blood culture media. By titrating the inoculum size and the concentration of SPS in an in vitro checkerboard assay, it was shown that concentrations of SPS >0.0008% prevented plasma coagulation. The 1:10 dilution of blood culture broth reduced the amount of residual SPS carried over to the TCT to a level (0.0005%) that did not impair plasma coagulation. The direct TCT inoculated with a 1:10 saline dilution of blood culture broth achieved 100% specificity and sensitivity within 4 h of inoculation without reducing the quality or quantity of coagulum.**

Following the widespread use of automated blood culture systems, the occurrence of an initial single positive blood culture bottle (BCB) with gram-positive cocci resembling staphylococci presents a common interpretative dilemma that may arise at any hour. Isolation of *Staphylococcus aureus* from blood cultures is usually indicative of significant clinical disease which requires prompt antibiotic treatment. More commonly, coagulase-negative staphylococci (CoNS) are isolated, and these are frequently found to be contaminants (14, 15). Rapid identification of *S. aureus* from blood cultures is essential for expeditious patient management, and several reports have described novel phenotypic and molecular methods for this purpose (5, 6, 11, 16). The tube coagulase test (TCT), however, remains the "gold standard" for *S. aureus* identification (1), and while the direct TCT has been reported to be highly specific, it has often lacked sensitivity when inoculated directly from BCB growing *S. aureus* (6, 13, 17).

We hypothesized that suboptimal sensitivity of TCT despite an abundance of organisms was due to the presence of sodium polyanetholesulfonate (SPS) in blood culture media acting as an anticoagulant and an agent that improves recovery of organisms in blood cultures (4, 9, 10).

We sought to improve the sensitivity of the TCT performed directly from positive blood culture bottles in which the Gram stain indicated the presence of bacteria resembling staphylococci when the bottle had flagged positive using the BacT/Alert (bioMérieux, Inc., Durham, N.C.) automated blood culture system.

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## MATERIALS AND METHODS

**Specimens.** During the two phases of this study, BacT/Alert standard and fastidious antitoxicity neutralization (FAN) media were received as aerobic, anaerobic, and pediatric formulations in BCB. In phase 1 of the study, the majority of bottles were standard BCB with only three aerobic FAN bottles, while in phase 2, only FAN BCB were tested. Gram-stained smears were examined from all bottles that signaled positive, and only those containing gram-positive cocci resembling staphylococci were included in this evaluation. All BCB were monitored by the Classic BacT/Alert automated blood culture system (bioMérieux Inc., Durham, N.C.). BCB may have been part of a paired collection, but for this study, each bottle was treated individually if the study criteria were fulfilled.

**Direct tube coagulase test.** The direct tube coagulase test was performed in 100- by 12-mm Pyrex glass tubes containing 1 ml of 10% pooled human plasma (fresh frozen plasma; Australian Red Cross Blood Transfusion Service, Sydney, Australia) containing the anticoagulant Adsol (Baxter Healthcare, Australia).

Two coagulase tubes were inoculated in parallel, the first with 4 drops (0.1 ml) of broth directly from the blood culture bottle (TCTdir) and the second with 4 drops (0.1 ml) from a 1:10 dilution of the broth (TCTsal), prepared by suspending 10 drops (0.25 ml) of blood culture broth in 2.5 ml of 0.9% saline. Both plasma tubes were examined after 4 h of aerobic incubation at 35°C. All tubes were then incubated overnight at room temperature and reexamined. The test was recorded as positive if a clot was observed at either time.

In phase 1, parallel testing of TCTdir and TCTsal was performed, while in phase 2, coagulase tubes were inoculated using only the TCTsal method.

**Confirmation of identity of *S. aureus*.** Staphylococci subcultured on blood agar from the BCB were tested for free coagulase production as the benchmark method by inoculating a single colony into 1 ml 10% human plasma incubated and examined as described above. All staphylococcal cultures were also tested for bound coagulase and protein A using a latex coagglutination test (Slidex; bioMérieux, Inc., Durham, N.C.).

**Viable count of positive blood culture bottles.** Three randomly selected blood cultures fulfilling the test criteria were serially diluted in 10-fold stages, and counts of viability were estimated from 100-μl aliquots subcultured on blood agar plates.

**Checkerboard titration of anticoagulant concentration and inoculum size on clot formation.** Equal volumes (50 μl) of serial 10-fold dilutions of *S. aureus* suspensions ($2 \times 10^9$ cells/ml) were mixed with serial twofold dilutions of 0.05%

TABLE 1. Number and percentage of positive results from direct TCT

| Identity of isolates in each phase | No. of positive results (%) | | | |
|---|---|---|---|---|
| | TCTdir | | TCTsal | |
| | 4-h incubation | Overnight incubation | 4-h incubation | Overnight incubation |
| Phase 1 ($n$ = 140)[a] | | | | |
| *S. aureus* ($n$ = 45) | 28 (62)[b] | 41 (91) | 40 (89) | 44 (98) |
| CoNS ($n$ = 95) | 0[b] | 0 | 0 | 0 |
| Phase 2 ($n$ = 36)[a] | | | | |
| *S. aureus* ($n$ = 19) | NA | NA | 19 (100) | 19 (100) |
| CoNS ($n$ = 17) | | | 0 | 0 |

[a] Number of samples tested by direct or saline TCT.
[b] Number of positive test results (percentage of confirmed *S. aureus* isolates in each phase).
[c] NA, no result, as only saline TCT was performed in phase 2.

SPS (Sigma Aldrich, St. Louis, Mo.) in a checkerboard array. The resulting 100-μl inoculum was added to a coagulase tube and read after 2 and 4 h of incubation at 35°C and after overnight incubation at room temperature. Clot quantity was arbitrarily graded from 1 to 4 by estimating the volume of the plasma solution replaced by the clot. This meant 25%, 50%, 75%, and 100% of the plasma volume occupied by a clot was recorded as grades 1 to 4, respectively. If there was no clot, it was scored as grade 0.

## RESULTS

The viable counts of staphylococci in the sample of bottles tested were within the range of $10^{11}$ to $10^{12}$ CFU per liter. The inoculum used in TCTdir was therefore estimated to be in the range of $10^7$ to $10^8$ CFU, and in TCTsal, it was estimated to be 1/10 of that inoculum. Following dilution of the BCB inoculum 1:10 in TCTdir and 1:100 in TCTsal, the final predicted concentrations of SPS carried over in the inocula were 0.005% and 0.0005%, respectively.

In phase 1, a total of 137 episodes representing 140 blood culture bottles (101 aerobic, including 3 FAN, and 39 anaerobic bottles) in which the Gram stain indicated probable staphylococci were examined. All samples grew staphylococci, of which 95 (68%) were CoNS and 45 (32%) were *S. aureus* isolates. The results of the tube coagulase tests are summarized in Table 1. All tests were performed prospectively without knowledge of the final identification. After 4 h of incubation, 40/45 (89%) *S. aureus* isolates were correctly identified by TCTsal, with 44/45 (98%) correctly identified after overnight incubation. Only 28/45 (62%) *S. aureus* isolates were correctly identified by TCTdir after 4 h of incubation, and 41/45 (91%) were correctly identified after overnight incubation.

There were no false-positive results with either the TCTsal or TCTdir after 4 h or overnight incubation. One isolate of *S. aureus* with a negative TCT by direct testing appeared to lack free coagulase on confirmatory testing using a single colony. This strain showed positive coagglutination using the Slidex (bioMérieux) test and a positive DNase test result (DNase CM321; Oxoid, Basingstoke, United Kingdom).

At the completion of phase 1, the routine BacT/Alert BCB received by our laboratory was changed from standard bottles to FAN bottles. As there were only three FAN bottles examined in phase 1, an additional 36 bottles were examined using only the TCTsal protocol, which had proved to be superior in phase 1. Nineteen of the 36 FAN bottles (53%) in phase 2 contained *S. aureus*, all of which were detected at 4 h by the TCTsal test. The results are also summarized in Table 1.

The interactive effects of the inoculum size and the SPS concentration on plasma clot formation are shown in Table 2. The strongest clots (grade 3 or 4) in the TCT were obtained and recognized earlier from those tubes with the greater inocula and the lowest SPS concentration. Clot formation was largely inhibited until the final SPS concentration in the plasma

TABLE 2. Checker board titration of SPS concentration and inoculum size for direct tube coagulase testing

| Final dilution of SPS (0.5%) | Grade of plasma clot[a] for *S. aureus* suspension[b] | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $2 \times 10^9$ CFU/liter[c] ($10^5$ organisms)[d] | | | $2 \times 10^8$ CFU/liter ($10^4$ organisms) | | | $2 \times 10^7$ CFU/liter ($10^3$ organisms) | | | $2 \times 10^6$ CFU/liter ($10^2$ organisms) | | | $2 \times 10^5$ CFU/liter (10 organisms) | | | 0 (saline) | | |
| | 2 h | 4 h | O/N | 2 h | 4 h | O/N | 2 h | 4 h | O/N | 2 h | 4 h | O/N | 2 h | 4 h | O/N | 2 h | 4 h | O/N |
| 1:2[c] | 0[a] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1:8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1:32 | 1 | 1 | 1 | 1 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1:64 | 3 | 3 | 3 | 4 | 4 | 4 | 2 | 3 | 3 | 0 | 2 | 3 | 0 | 0 | 3 | 0 | 0 | 0 |
| 1:128 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 3 | 0 | 4 | 4 | 0 | 0 | 4 | 0 | 0 | 0 |
| 0 (saline) | 4 | 4 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 0 | 4 | 4 | 0 | 0 | 4 | 0 | 0 | 0 |

[a] Clot size was graded by estimating the volume of coagulated plasma in the tube as a proportion. Thus, grades 1 to 4 represent plasma clots occupying 25%, 50%, 75%, and 100% of the plasma solution, respectively. Absence of a plasma clot was scored as 0% (grade 0). Grades were recorded after 2-h, 4-h, and overnight (O/N) incubations.
[b] Strain used in the duplicate assays was a clinical isolate from phase 2.
[c] Each tube was inoculated with 50 μl of bacterial suspension ($10^5$ to 10 organisms) and 50 μl SPS dilution (final dilution, 1:2 to 1:128 of 0.05% SPS).
[d] Actual inoculum.

was diluted 1:64, using 50 μl of the 1/32 dilution of the 0.05% SPS solution; however, grade 4 clots were also recorded at 4 h by inocula as small as 100 colony-forming organisms in the presence of a 1:128 dilution of the 0.05% SPS solution.

## DISCUSSION

It has been well documented that patient care is improved and costs are reduced if reliable rapid coagulase results are available for blood cultures containing staphylococci (2, 8, 18). Previous studies (7, 12, 13, 17) have examined rapid methods of staphylococcal identification from automated blood culture instruments other than the BacT/Alert with varying success.

We aimed to incorporate the direct tube coagulase test as part of the routine identification of staphylococcus from blood cultures. By utilizing the method of using undiluted blood culture broth as the inoculum as described previously by Speers et al. (17), we were able to test our hypothesis empirically and in parallel by using a 1:10 saline dilution of the blood culture broth with minimal change to laboratory workflow in phase 1 of the study.

As both phases of this study were included in the routine laboratory workflow, all laboratory staff were trained to read and record the direct tube coagulase results. Multiple observers in this study may have introduced the possibility of observer variation, particularly when clot formation was low grade. Thus, the lower percentage of positive results in phase 1 using the TCTdir method may have been due to impaired recognition of small clots due to the higher blood and charcoal concentrations in those test conditions.

Another explanation for the lower percentage of positive results using the TCTdir method relates to the concentration of SPS carried over from the blood culture bottle into the coagulase tube. SPS is a polyanionic anticoagulant, which, among other functions, prevents blood clotting by inhibiting complement (9, 10). The inhibition of coagulation by SPS in the checkerboard titration was virtually eliminated when the 0.05% SPS was diluted 1:64 (0.0008% SPS), although there was a noticeable inhibitory effect using a 1:32 dilution (0.0016% SPS). The predicted carryover level of 0.005% or 0.0005% SPS may be an overestimation because residual SPS in an inoculated BCB could be influenced by the extent of SPS binding with blood, plasma proteins, antimicrobials, and possibly other compounds. There may be other inhibitors of coagulation that remain unrecognized.

Plasma clots were recognized earlier using the diluted sample in both phases, which supports the hypothesis that the insensitivity of the direct tube coagulase test in the past has been due to carryover of inhibitors of coagulation such as SPS rather than a paucity of staphylococci producing free coagulase. Moreover, the inocula used in the checkerboard assay showed that even inocula as low as 100 CFU could produce a grade 4 clot within 4 h in the absence of SPS. In practice, most inocula used in the TCTsal were probably about $10^6$ organisms, which were more than sufficient to give a positive TCT result within 4 h.

In this study, human plasma was used in the TCT. There is no reason to believe that the results would be different with rabbit plasma, as recommendations for the use of either have been made (1, 3).

The TCTsal is both sensitive and specific for identifying *S. aureus* from a single positive bottle within 4 h of incubation. The test can be set up at any time without batching samples and creates no additional technical demands for existing laboratory staff. Furthermore, this test would greatly reduce the numbers of samples that might be subsequently tested for the presence of the *mecA* gene as an additional guide to early effective therapy of *S. aureus* bacteremia.

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