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Direct Testing of Blood Cultures for Detection of Streptococcal Antigens

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A direct, rapid, and simple method for the detection of streptococcal antigens of Lancefield groups A, B, C, D, and G from blood cultures was developed by using a coagglutination test. Fifty-five clinical specimens and 117 simulated blood cultures containing gram-positive cocci were tested. Out of 6,261 clinical blood cultures screened, 55 cultures from 53 patients were positive, with organisms resembling streptococci, by Gram stain. Of these cultures, 78% (43 of 55) were pure cultures of streptococci, and 22% (12 of 55) were mixed with at least one other organism. Of the 43 pure cultures only, correct reactions were obtained (grouping correctly or giving no cross-reactions, or both) with 86% (37 of 43) of the isolates, 12% (5 of 43) exhibited cross-reactions, and 2% (1 of 43) gave false-negative reactions. All of the cross-reacting isolates were *Streptococcus pneumoniae*, which reacted with the group C reagent, and the false-negative reaction occurred with a *Streptococcus bovis* isolate. However, by using a direct modified bile solubility test, the correct identification of the *S. pneumoniae* isolates was obtained. Therefore, by using the modified bile solubility test in conjunction with the direct grouping method, 98% (42 of 43) of the isolates in pure culture could be identified accurately and rapidly after the detection of a positive Gram stain. Correct grouping reactions were obtained with 83% (10 of 12) of the mixed blood cultures, and false-negative results occurred with 17% (2 of 12) of them. Both cultures contained an enterococcus and a gram-negative rod. Of the 117 simulated blood cultures, there was only one incorrect grouping reaction; this occurred with an *S. bovis* isolate that cross-reacted with the group C reagent. The direct grouping reaction was positive when blood cultures contained a minimum of 1×10^8 to 8×10^8 colony-forming units per ml. In general, this procedure provided information on the identification of the organism 24 h earlier than by conventional identification methods.

Today, the emphasis in the clinical microbiology laboratory is on the rapid detection and identification of organisms. Blood cultures and other body fluids are some of the most important specimen types processed in the laboratory, since results can significantly influence the therapy and final outcome of the patient. Therefore, many investigators have focused their attention on improving reporting time with these specimens (1, 5, 8, 11, 12). Direct testing of fluids by counterimmunoelectrophoresis and coagglutination has proven to be a valuable asset in the early detection of organisms (1, 5, 8, 12). Compared with coagglutination, counterimmunoelectrophoresis is more cumbersome and time-consuming to perform. Additionally, in a study by Webb et al. (12), coagglutination proved more rapid and, in some instances, more sensitive than counterimmunoelectrophoresis for the detection of streptococcal antigens in cerebrospinal fluid.

Thus far, the coagglutination method has only been used to directly test for group B streptococcal antigen in a very limited number of clinical blood cultures (8). Hence, by expanding on this concept, the objective of this study was to develop a method in which coagglutination could be used to directly test blood cultures for streptococcal groups A, B, C, D, and G.

MATERIALS AND METHODS

Simulated blood cultures. (i) Inoculation of cultures. Stock isolates collected from clinical specimens and frozen at −70°C in fetal calf serum were inoculated into blood cultures for direct coagglutination testing. The organisms were transferred to blood agar twice before testing and were then inoculated into 2 ml of Todd-Hewitt broth and incubated aerobically for 12 to 18 h at 35°C. Subsequently, 1 ml of each culture was inoculated into 7-day-old negative blood cultures containing 10 ml of blood in 90 ml of tryptic soy broth, with 0.025% sodium polyanetholesulfonate under vac-

uum with CO_2 (Difco Laboratories, Detroit, Mich.), and incubated overnight at 35°C.

(ii) Groups A, B, C, and G. In the development of the direct coagglutination procedure, stock cultures of beta-hemolytic streptococcal groups A, B, C, and G as well as alpha-hemolytic and nonhemolytic streptococci were seeded into blood cultures and tested. Non-beta-hemolytic streptococci were included to determine the frequency of cross-reactions, if any. After overnight incubation of the seeded blood cultures, 2 ml was removed, Gram stained, placed in a sterile tube, and centrifuged for 10 min at 1,000 × *g*. The supernatant was removed and placed in a boiling water bath for 3 min. This suspension was centrifuged for 10 min at 1,000 × *g*. The resulting supernatant was tested by using the Phadebact Streptococcus Test kit (Pharmacia Diagnostics, Piscataway, N.J.) for groups A, B, C, and G according to the manufacturer's directions.

(iii) Group D. An alternate procedure was developed for group D streptococci due to preliminary trials in which very weak or no positive groupings were obtained with these isolates. After overnight incubation of seeded blood cultures, 5 ml was removed and placed in a sterile tube. The specimen was centrifuged at 1,000 × *g* for 30 s to sediment most of the erythrocytes. The supernatant was removed and centrifuged for 10 min at 1,000 × *g* to pellet the organisms. Sterile water (5 ml) was added to the bacterial pellet, and the cell suspension was blended vigorously with a Vortex mixer for 30 s to lyse any remaining erythrocytes. This suspension was centrifuged at 1,000 × *g* for 10 min, and the supernatant was discarded. The pellet was resuspended in 1 ml of sterile water, blended with a Vortex mixer for 30 s, boiled in a water bath for 3 min, and filtered (5 μm) to remove any particulate matter. The filtrate was tested with the Phadebact streptococcal group D coagglutination reagents according to the manufacturer's directions.

(iv) Sensitivity. Cell suspensions were standardized to 10^7 colony-forming units (CFU)/ml by using the Autobac MTS (Pfizer, Inc., New York, N.Y.) and subsequently were diluted in saline to obtain the desired concentration (10^3 CFU/ml). All bacterial suspensions were checked quantitatively by plate counts to establish the exact concentration at the time of inoculation. Organisms (10^3 CFU) were inoculated into negative blood cultures, incubated at 35°C, and tested by direct coagglutination at 2-h intervals until a positive grouping was obtained; then, testing by Gram stain and by a quantitative plate count was performed on the positive culture.

Clinical isolates. Clinical blood specimens were collected for culture by standard sterile technique. Twenty milliliters of blood was collected from adults, and half of the specimen was injected into each of two 100-ml blood culture bottles (tryptic soy broth; Difco Laboratories). One bottle of the set was vented with a sterile cotton-plugged needle to provide aerobic conditions. Blood (3 to 5 ml) was collected from pediatric patients and injected into a 50-ml blood culture bottle. All of the bottles were incubated at 35°C until they were found to be positive or for a maximum of 7 days. The cultures were checked daily macroscopically for evidence of growth, Gram stained, and subcultured to chocolate agar at 12, 24, and 72 h.

Gram stains of these clinical blood cultures were examined for the presence of gram-positive cocci arranged in pairs and chains, which is consistent with streptococcal arrangements. Also, chocolate subcultures of blood cultures were examined for the presence of organisms resembling streptococci. If such organisms were observed by either Gram stain or subculture, the direct coagglutination procedure, using material from the blood culture broth, was performed. Since both of the coagglutination procedures (the one for groups A, B, C, and G and the one for group D) were to be performed, a combination of the two methods described above was developed. From all positive cultures, 5 ml of blood was removed and centrifuged at 1,000 × *g* for 30 s. The supernatant was removed and centrifuged for 10 min at 1,000 × *g*. The direct coagglutination procedure for groups A, B, C, and G was performed only on the resulting supernatant, and the group D procedure was performed only on the resulting bacterial pellet as previously described (Fig. 1).

A modified bile solubility test for the rapid identification of *Streptococcus pneumoniae*, as described by Murray (10), was also performed on all clinical blood cultures containing gram-positive cocci that resembled streptococci.

Along with these direct methods, conventional methods were performed on the subcultured organisms for a final identification and comparison of results (6).

RESULTS

Simulated blood cultures. (i) Antisera for groups A, B, C, and G. A total of 109 different isolates were tested for groups A, B, C, and G, including 10 different species of streptococci. These isolates included: beta-hemolytic streptococcal group A (25 isolates), group B (14), group C (4), group G (4), neither group A, B, C, nor G (3), enterococci (34), *Streptococcus bovis* (9), viridans streptococci (14), *S. pneumoniae* (1), and an anaerobic gram-positive coccus (1). Of these isolates, 43% (47 of 109) belonged to group A, B, C, or G, and all were identified correctly by the direct coagglutination test and gave no cross-reactions. Fifty-seven percent (62 of 109) of the isolates were streptococci other than group A, B, C, or G, and only 1 of the 62 showed a cross-reaction. The one discrepant isolate was an *S. bovis* isolate that cross-reacted with group C.

(ii) Group D antisera. The following eight organisms were tested: enterococci (3 isolates), *S. bovis* (3), *S. pneumoniae* (1), and viridans streptococcus (1). All enterococcal and *S. bovis* isolates coagglutinated with the group D reagent, whereas the *S. pneumoniae* and viridans streptococcal isolates showed no cross-reactivity.

(iii) Sensitivity. Blood cultures inoculated with organisms (10^3 CFU/ml; final concentration, 10 CFU/ml) had positive direct coagglutination reactions between 10 and 18 h of incubation. The

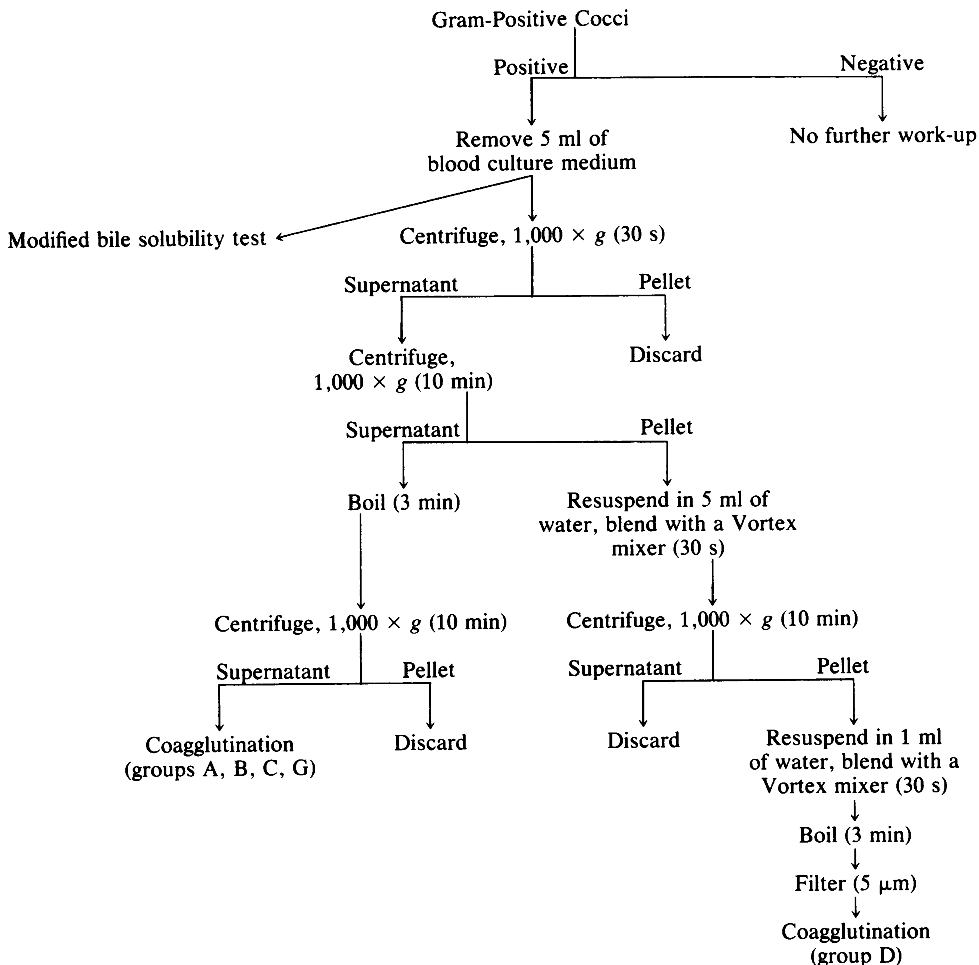

FIG. 1. Identification of streptococci from clinical blood cultures.

minimum concentration of the culture at the time of detection ranged from 9.0×10^7 to 8.7×10^8 CFU/ml, depending on the streptococcal species present (Table 1). In each instance, when the direct coagglutination reaction turned positive, the Gram stain result was also positive.

Clinical isolates. Out of 6,261 clinical blood cultures screened, 55 cultures from 53 patients were found to be positive, with organisms resembling streptococci, by Gram stain. Since one of these cultures had multiple streptococcal organisms present, there were actually a total of 56 different streptococcal isolates tested. The isolates consisted of nine different streptococcal species including beta-hemolytic, alpha-hemolytic, and nonhemolytic streptococci as well as an anaerobic gram-positive coccus (Tables 2 and 3). Eighty-five percent (47 of 55) of the culture isolates were initially found to be positive by Gram stain or macroscopic inspection, and 15% (8 of 55) were initially found to be positive by blind subculture. Sixteen percent (9 of 55) of the cutures were positive at 12 h, 45% (25 of 55) at 18 to 24 h, 20% (11 of 55) at 48 h, 5% (3 of 55) at 72 h, and 13% (7 of 55) of the cultures were found to be positive after 72 h of incubation.

Seventy-eight percent (43 of 55) of the cultures tested were pure cultures of streptococci, and 22% (12 of 55) of the cultures also contained organisms other than streptococci. The results of these two groups of cultures will be discussed separately.

(i) Pure cultures. Forty-nine percent (21 of 43) of the pure cultures tested contained streptococci belonging to group A, B, C, D, or G. The direct coagglutination test correctly identified 95% (20 of 21) of these isolates. The one (1 of 21) isolate not identified correctly by the direct coagglutination test was an *S. bovis* isolate which gave a false-negative reaction. No cross-

TABLE 1. Sensitivity data: simulated cultures

Organism	Direct coagglutination positive reaction (h)	Avg concn[a] (CFU/ml) at time of detection
Beta-hemolytic streptococcus:		
Group A	18	1.6×10^8
Group B	12	9.0×10^7
Group C	18	2.1×10^8
Group G	16	4.3×10^8
S. bovis	10	8.7×10^8
Enterococcus	10	2.3×10^8

[a] At time zero, blood cultures were seeded with organisms to contain approximately 10 CFU/ml.

reactions were seen with any of these 21 isolates. One of the cultures tested demonstrated gram-positive cocci in chains by direct Gram stain and gave a positive group D reaction. On subculture, the organism was nonviable. However, cultures that had been collected 2 days before and 3 days after this culture from the same patient also gave a positive group D reaction and subsequently grew out an enterococcus; therefore, the antigen was probably correctly detected in the nonviable culture. The patient was on three different antimicrobial agents (penicillin G, cefoperazone, and amikacin) at the time that these cultures were taken; therefore, this may be a possible explanation for the nonviability of the organism.

The remaining 51% (22 of 43) of the pure cultures contained streptococci other than group A, B, C, D, or G. With 77% (17 of 22) of these organisms, no cross-reactions were detected. The five (5 of 22) isolates giving cross-reactions were *S. pneumoniae*, and all strongly cross-reacted with the group C reagent. However, in each case these cultures were positive by the modified bile solubility test.

TABLE 2. Direct coagglutination testing of blood cultures: pure cultures

Organism	No. of isolates	Correct reactions
Beta-hemolytic streptococcus		
Group A	2	2
Group B	6	6
Group F	1	1
Group G	1	1
S. bovis	5[a]	4
Enterococcus	6	6
S. pneumoniae	5[b]	
Viridans streptococcus	16	16
Anaerobic gram-positive coccus	1	1

[a] One false-negative reaction occurred.
[b] All of the isolates cross-reacted with group C, and all were positive by the modified bile solubility test.

TABLE 3. Direct coagglutination testing of blood cultures: mixed cultures

Organism	No. of isolates	Correct reactions
Enterococcus and:		
Enterobacter cloacae	2[a]	1
Escherichia coli	1	1
Klebsiella pneumoniae	1[b]	
Staphylococcus aureus	1	1
Torulopsis glabrata	1	1
Escherichia coli and *Proteus mirabilis*	1	1
Viridans streptococcus and:		
Escherichia coli	1	1
Klebsiella pneumoniae	1	1
Staphylococcus aureus	1	1
Escherichia coli and *Klebsiella pneumoniae*	1	1
Viridans streptococcus no. 2 and *Staphylococcus epidermidis*	1	1

[a] One false-negative reaction occurred.
[b] Non-interpretable reaction, both the control and the test reacted to the same degree.

(ii) Mixed cultures. The 12 mixed cultures tested included 7 containing enterococci and 5 containing viridans streptococci. Each of these cultures also contained one or more nonstreptococcal organisms, and one culture contained two different viridans streptococci along with a *Staphylococcus epidermidis* isolate (Table 3). Eighty-three percent (10 of 12) of the cultures gave the appropriate reactions, grouping correctly or giving no cross-reactions (or both). Seventeen percent (2 of 12) of these mixed cultures gave incorrect reactions. Each of these two cultures contained an enterococcus isolate and another organism. One culture was mixed with *Enterobacter cloacae* and gave a false-negative reaction. The other culture was mixed with *Klebsiella pneumoniae* and gave a non-interpretable reaction (both the control and the test reacted to the same degree).

DISCUSSION

Rapid identification of organisms is important, especially in cases of positive blood cultures or spinal fluids, because it enables the clinician to better evaluate and treat the patient. For this reason, rapid identification of organisms has

been a major area of research in the microbiology laboratory over the past few years. This is evidenced by the numerous studies which have attempted to solve this problem by testing the reliability of different methods of bacterial identification (e.g., latex agglutination, coagglutination, enzyme-linked immunosorbent assay, counterimmunoelectrophoresis) directly on body fluids and blood cultures (1, 5, 8, 11, 12).

In our study the coagglutination test was chosen because it has been shown to be a very rapid, accurate, and easy test to perform for identifying streptococci (3, 7, 9). We wanted to expand on previously reported studies (8, 11, 12) to directly test for streptococcal antigens in blood cultures other than just group B and to include patients of all age groups rather than just infants. We developed a method for direct coagglutination testing for streptococcal groups A, B, C, D, and G by using simulated blood cultures and subsequently used this method to test clinical cultures. The clinical cultures tested included any blood culture with organisms resembling streptococci on Gram stain. Two different portions of the blood culture specimen were used for testing. The group-specific antigen of streptococcal groups A, B, C, and G is a cell surface polysaccharide (4); thus, it readily diffuses into the broth medium. Therefore, the supernatant from the blood culture broth was successfully used to test for these groups. With group D streptococci, the group-specific antigen is a lipoteichoic acid closely associated with the cell membrane (4); hence, the bacterial pellet was used for testing. As described by Doskeland and Berdal (5), a boiling step was added which eliminated most cross-reactions. They also observed that EDTA aided in sedimenting denatured protein that would otherwise remain in suspension as a gel and thus trap antigen. We found, however, that the addition of this reagent to the supernatant did not improve bacterial antigen detection; therefore, the use of EDTA was not included in our procedure.

According to the manufacturer's directions for the Phadebact Streptococcus Test for groups A, B, C, and G, if certain bacteria other than beta-hemolytic streptococci are tested, an improper result might be obtained. Since no major cross-reaction problems occurred with the non-beta-hemolytic streptococci tested for groups A, B, C, and G in the simulated blood cultures, it was concluded that there was no need for hemolysis determination of the organisms before testing. This enables identification time to be reduced considerably, since it takes a minimum of 4 h to determine the hemolysis pattern of an organism after subculture.

The pure cultures tested showed 86% (37 of 43) correct identifications, 12% (5 of 43) cross-reactions, and 2% (1 of 43) false-negative reactions. All of the cross-reactions were due to *S. pneumoniae* isolates which reacted with the group C streptococcal reagents. This is in accordance with the known similarities between the antigens of these two organisms (3). However, since the modified bile solubility test (10) was positive with all these isolates, this test, used in conjunction with the direct coagglutination test, gave a 98% (43 of 44) accuracy of identification. The false-negative result occurred with an *S. bovis* variant identified by Facklam's scheme (6). Conventional coagglutination testing of this organism also proved negative, possibly due to the small amounts of group D antigen present in some strains of enterococci and *S. bovis* (6).

Only 17% (2 of 12) of the mixed clinical cultures exhibited false-negative reactions. These false-negative reactions occurred with two cultures of enterococci, one mixed with *E. cloacae* and one mixed with *K. pneumoniae*. The culture mixed with *K. pneumoniae* actually gave a non-interpretable result but, as per the manufacturer's directions, was considered to be negative. These false-negative reactions may have been due to interfering substances produced by the gram-negative rods that either blocked or caused a nonspecific reaction to occur or due to too low a concentration of streptococci. Therefore, on blood cultures with both gram-positive cocci and gram-negative rods present on Gram stain, there is a possibility that a false-negative reaction could occur.

The results also show that the majority (61%) of the positive blood cultures were detected in 24 h. Additionally, most of the blood cultures were initially found to be positive by Gram stain or by macroscopic inspection (85%) rather than from blind subcultures (15%). This emphasizes that the direct coagglutination procedure is of major benefit in providing information about the organism 24 h earlier than would otherwise be possible in the majority of the cultures.

Using simulated blood cultures, we showed that the direct coagglutination procedure is capable of detecting organisms when a minimum of 9.0×10^7 to 8.7×10^8 CFU/ml are present, depending on the species of streptococci. This closely agrees with Leland et al. (9), who found that the group B streptococcal antigen is capable of being detected in the supernatant of a broth medium by coagglutination when 6×10^8 CFU/ml are present. Since one or more bacteria per oil immersion field are found by Gram stain when the count is $>10^5$ CFU/ml (2), the possibility exists that a positive Gram stain may result with a negative coagglutination reaction.

In this study we utilized a blood culture system which consisted of tryptic soy broth; however, clinical laboratories utilizing a different

blood culture medium should run preliminary experiments to ensure that the culture medium does not interfere with direct testing.

In conclusion, this direct coagglutination method for blood cultures is a quick, easy, and reliable test that can yield extremely valuable information about the organism's identification. This method should be expanded to include other body fluids such as cerebrospinal fluid and urine as well as the identification of organisms other than streptococci.

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