

Rapid Slide Coagglutination Test for Identifying and Typing Group B Streptococci

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A Cowan I strain of *Staphylococcus aureus* was labeled with either group B streptococcal grouping or typing antiserum. These antibody-labeled reagent cells (ARC) were used in a slide coagglutination test to identify and type group B streptococci from blood agar plates. All streptococci were also identified by the standard Lancefield capillary precipitin test. In a blind study, all 141 group B streptococci were correctly identified by the coagglutination grouping test. None of the 148 non-group B streptococci caused agglutination of ARC. The coagglutination grouping test required an acid extract prepared from only four colonies and could be completed less than 30 min after colonies were removed from plates. The coagglutination typing test correctly identified 98.6% of the types of the 141 group B streptococcal strains tested. At least 88.6% of these streptococci could be typed directly from blood agar plates within 5 min by the coagglutination typing test. The remaining 11.4% of the group B streptococci were acid extracted (less than a 30-min procedure), and the extract was used for coagglutination typing. Coagglutination typing can be performed with only four colonies. The coagglutination grouping and typing tests are inexpensive, rapid, reliable, and easy to perform.

The group B streptococcus *Streptococcus agalactiae* has been implicated as a causative agent in a wide variety of human infections. Included are meningitis, pneumonia, endocarditis, arthritis, empyema, abscesses, osteomyelitis, septicemia, and genitourinary tract infections (16). Neonatal infections are of particular concern because of their high incidence and mortality rate (1, 2, 7). Early diagnosis is imperative in neonatal infections to ensure prompt and appropriate therapy.

S. agalactiae has traditionally been identified by the Lancefield capillary precipitin (CP) reaction (15). This technique requires an 18- to 24-h culture period and a time-consuming extraction procedure and is, therefore, of limited value in neonatal infections. A variety of methods have been developed and used to simplify and speed the identification of group B streptococci. The 2-h hippurate hydrolysis test (10) and CAMP reaction as described by Darling (4) have been used for rapid presumptive identification. Fluorescent-antibody techniques (14) and counterimmunoelectrophoresis (8) have also been used for definitive identification, but the equipment and reagents necessary for these tests are not available in many laboratories.

Christensen et al. reported a method for the rapid and specific identification of streptococci

(3) using antibody-labeled *Staphylococcus aureus* cells (ARC). We have modified the procedure and used it to identify and type group B streptococci.

MATERIALS AND METHODS

Reagent cells. *S. aureus* reagent cells were prepared with modifications of the methods of Minor and Marth (12). Dialysis tubing (3.25 inches [approximately 8.3 cm] wide; S.S.D.C., Union Carbide, Chicago, Ill.) was cut into flat 90-mm disks. Filter paper was inserted between each disk, and the disks were autoclaved in a moist chamber at 121°C for 15 min. A sterile disk was placed on the surface of brain heart infusion (BHI; Difco) agar to cover the surface. A Cowan I strain of *S. aureus* was grown in BHI broth (Difco) at 35°C and transferred three or four times in BHI broth at 9- to 14-h intervals. Cells were washed three times in sterile phosphate-buffered saline (PBS; 1.096 g of Na₂HPO₄, 0.315 g of NaH₂PO₄·H₂O, and 8.5 g of NaCl per liter, pH 7.3) and resuspended in PBS to a 10% (vol/vol) concentration. A 0.1-ml quantity of cells was inoculated onto the disk on the BHI plate and spread with a sterile glass rod. Plates were incubated in an upright position at 35°C for 18 h.

The luxuriant growth of *S. aureus* cells was harvested by adding 1 ml of PBS to the plate, removing the cells with a sterile glass rod and pipette, and repeating the process. The cells were washed three times in PBS and resuspended in PBS with 0.5%

formaldehyde to a 10% concentration. After standing for 3 h at room temperature, the treated cells were washed three times and resuspended in PBS to a 10% concentration. This suspension was heated for 20 min in an 80°C water bath and resuspended in PBS with 0.002% Merthiolate to a final 10% concentration. Reagent cells could be stored for several months at 4°C.

ARC. The ARC were prepared with modifications of the methods of Edwards and Larson for the identification of group A streptococcus (5). Group B streptococcus grouping and typing antisera (types Ia, Ib, II, and III) were obtained from the Center for Disease Control, Atlanta, Ga. Reagent cells were labeled with individual antiserum by adding 0.035 ml of antiserum to 0.25 ml of cells and allowing the mixture to stand at room temperature for 2.5 h. The mixture was centrifuged, and the supernatant was discarded. These ARC were resuspended in 2 ml of PBS with 0.002% Merthiolate and 0.02 ml of 2% Procion brilliant blue, M-RS (Colab Laboratories, Chicago Heights, Ill.). The ARC were stable for at least 4 months when stored at 4°C.

Organisms and media. Stock cultures and fresh clinical isolates of group A, B, C, D, F, and G streptococci were obtained from the Wisconsin State Laboratory of Hygiene and Madison General Hospital. These isolates were identified by growth in 6.5% NaCl broth, bile esculin hydrolysis, hippurate hydrolysis, and the standard Lancefield CP test for grouping and typing (6, 15). Streptococci were transferred to 7.5% sheep blood agar plates (veal infusion base; BBL), incubated under 10% CO₂ at 35°C for 18 h, and then tested by the coagglutination procedure.

Coagglutination grouping procedures. The majority of the group B streptococci could be grouped only if a hot-acid extract was prepared. The grouping procedure was always attempted directly with whole cells initially and then with the extract if necessary. Care was taken to pick pure cultures and to subculture when necessary. Two 15-mm paraffin rings were applied to a glass slide, and 0.5 drop of 0.85% saline was added to each ring from a Pasteur pipette. Two colonies were suspended in the saline, and 1 drop of the grouping ARC or unlabeled reagent cells was delivered to each ring from a Pasteur pipette. The slide was rocked continuously for up to 4 min, and macroscopic agglutination was observed.

The second method involved removing well-isolated colonies from the plate, preparing a hot-acid extract of the cells, and testing the extract with ARC. This extract was prepared by emulsifying at least four colonies in 3 to 4 drops of saline in a test tube (12 by 75 mm) and adding a drop of 0.04% metacresol purple and sufficient 0.2 N HCl to make the suspension a salmon pink color. The tube was placed in a boiling water bath for 10 min, neutralized with 0.2 N NaOH, and centrifuged until a clear supernatant was obtained. The test was performed by delivering 0.5 drop of the extract supernatant to two paraffin rings on a glass slide. A drop of the ARC was added to one ring, and a drop of unlabeled reagent cells was added to the second ring. The slide was rocked continuously for up to 4 min, and macroscopic agglutination was observed.

Coagglutination typing procedures. Most group B streptococci could be typed directly from plates without preparing an extract. Five 15-mm paraffin rings were applied to a glass slide, and a drop of 0.85% saline was delivered to each ring. Four similar streptococcal colonies were simultaneously collected on a bacteriological loop and suspended to a uniform turbidity in each of the saline-containing rings. A drop of one of the four types of ARC was added to four rings, and a drop of unlabeled reagent cells was added to the fifth. The slide was rocked continuously for 3 min, although macroscopic agglutination was usually observed after 30 s. If the streptococci failed to agglutinate any of the ARC or a very weak agglutination reaction occurred, then a hot-acid extract (described above) was prepared and the coagglutination test was repeated with the extract. Agglutination developed more slowly with an extract and was of a finer texture than that observed with whole cells.

RESULTS

Coagglutination grouping. Initially the coagglutination grouping procedure was performed by adding ARC to streptococcal cells suspended in saline on slides. Only 14% of the 141 group B streptococci tested in this manner caused ARC agglutination. Non-group B streptococci frequently autoagglutinated in the saline control.

Because of the poor results obtained with the above method, the procedure was modified. Streptococci were removed from blood agar plates, and acid extracted, and the extract was tested with reagent cells labeled with group B antiserum. Table 1 shows the coagglutination results obtained with ARC and streptococcal cell extracts derived from the modified hot-acid extraction method. All group B streptococcal

TABLE 1. Specificity of the coagglutination test for identifying group B streptococci

Lancefield group	Stock strains		Clinical strains	
	No. positive by coagglutination ^a	No. positive by Lancefield CP test ^b	No. positive by coagglutination ^a	No. positive by Lancefield CP test ^b
A	0	5	0	38
B	96	96	45	45
C	0	10	0	16
D	0	25		
F	0	12	0	12
G	0	10	0	20

^a Results were obtained with reagent cells labeled with group B antiserum and tested with whole streptococcal cells or by the modified hot-acid extraction technique.

^b Results were obtained with standard Lancefield hot-acid extracts and standard grouping antisera.

isolates produced strong ARC agglutination. These ARC failed to agglutinate with extracts from any of the 148 non-group B streptococci tested. Neither the group B nor the non-group B streptococci agglutinated the unlabeled reagent cells. Cell extraction and coagglutination grouping could be completed in less than 30 min. Thus, 100% of the group B streptococci were correctly identified if the coagglutination test was performed on cell extracts. The CP results were obtained with standard Lancefield hot-acid extracts and standard grouping antisera.

Coagglutination typing. When the typing procedure was performed, 88.6% of the group B strains tested could be typed directly with whole cells without acid extraction. The ARC agglutination patterns observed with different types of group B streptococci are shown in Table 2. Streptococcal types Ib and II produced strong agglutination reactions only with homologous reagent cells. Type Ic streptococci gave strong agglutination reactions with both type Ia and Ib reagent cells, and type II(Ib) streptococci gave strong agglutination reactions with both type Ib and II reagent cells (16). Streptococcal types Ia and III strongly agglutinated homologous reagent cells and frequently weakly agglutinated type III and Ia reagent cells, respectively. To correctly establish types by the coagglutination procedure, these weak agglutination reactions should be disregarded when strong agglutination reactions are simultaneously observed* with type Ia or III reagent cells.

When nontypable (NT) strains (13) were tested by the whole-cell coagglutination procedure, ARC did not agglutinate or, rarely, weak ARC agglutination occurred (Table 2). Sixteen

TABLE 2. Coagglutination patterns obtained with different types of group B streptococci

Agglutination type	Patterns ^a with ARC			
	Anti-Ia	Anti-Ib	Anti-II	Anti-III
Ia	+	-	-	±
Ib	-	+	-	-
Ic	+	+	-	-
II(Ib)	-	+	+	-
II	-	-	+	-
III	±	-	-	+
NT ^b	-	-	-	-

^a +, Strong agglutination of ARC; -, no agglutination of ARC; ±, weak agglutination of ARC frequently occurred and no agglutination rarely occurred.

^b Nontypable (NT) strains do not agglutinate ARC either before or after acid extraction.

strains of group B streptococci were NT by coagglutination when using whole cells (Table 3). These strains were treated with the modified hot-acid extract method, and the coagglutination test was repeated. Interpretation of agglutination patterns was as described in Table 2. After extraction and testing with ARC, nine strains remained NT, one strain was type II, and six strains were type III.

Table 4 shows the agreement of typing results obtained by the coagglutination procedure using whole streptococcal cells and the modified hot-acid extraction procedure with typing by the standard Lancefield hot-acid extraction test. Overall 98.6% of the types established by the coagglutination procedure agreed with types established by the CP test. When clinical isolates were typed, 100% agreement was obtained between the two methods. There was 100% agreement between the two methods for type Ia, Ic, II(Ib), II, and NT streptococci. Disagreement between the two methods occurred with two stock cultures. Strain 216 was type Ib by the CP test, but Ib and II antigens were detected by the coagglutination procedure. Strain 437 was type III by the CP test, but type III and II antigens were detected by the coagglutination procedure. These discrepancies could be due to cross-reacting antibodies in the antisera, since the antisera were prepared for

TABLE 3. Number of group B streptococci that were typable by the coagglutination technique with whole cells or only after extraction

Lancefield type	No. typable by given method	
	From whole cells ^a	From extract ^b
Ia	34	-
Ib	16	-
Ic	2	-
II(Ib)	3	-
II	24	1
III	46	6
NT	16 ^c	9
Total	125	16

^a Number of strains giving a positive coagglutination reaction when tested with whole cells from four colonies.

^b Number of strains that gave a negative or weakly positive coagglutination reaction when tested with whole cells from four colonies and had to be retested with the modified hot-acid extraction technique.

^c Sixteen strains were found to be nontypable (NT) when tested with whole cells from four colonies; when these same 16 strains were retested after acid extraction, 6 were found to be type III and 1 was found to be type II. The remaining 9 were NT.

TABLE 4. Agreement of group B streptococcal types established by the coagglutination test with types established by the Lancefield CP test

Lancefield type	Stock strains		Clinical strains	
	No. positive by coagglutination ^a	No. positive by Lancefield CP test ^b	No. positive by coagglutination ^a	No. positive by Lancefield CP test ^b
Ia	22	22	12	12
Ib	8 ^c	9	7	7
Ic	2	2	0	0
II(Ib)	0	0	3	3
II	18	18	7	7
III	38 ^d	39	13	13
NT	6	6	3	3
Total	94	96	45	45

^a Number of strains that agglutinated reagent cells labeled with group B typing antiserum when tested with whole streptococcal cells or the modified hot-acid extraction technique.

^b Number of strains of the various Lancefield types that precipitated in a standard Lancefield hot-acid extraction procedure with standard typing antisera.

^c One strain was type Ib by the CP test, but Ib and II antigens were detected by the coagglutination test.

^d One strain was type III by the CP test, but III and II antigens were detected by the coagglutination test.

use in the Lancefield CP tests. These discrepancies could not be attributed to multiple strains or different types within the same culture.

DISCUSSION

On the basis of these results, the coagglutination grouping procedure appears to be a reliable, rapid, and easy test for the identification of group B streptococci. The coagglutination grouping procedure was specific for group B streptococci when 141 group B streptococci and 148 non-group B streptococci were tested. Once isolated beta-hemolytic streptococci appear on media, group B streptococci can be accurately identified by the coagglutination test in less than 30 min, whereas grouping by the Lancefield CP technique takes at least 18 h. With limited experience, a microbiologist can interpret the coagglutination reactions more easily than CP reactions. All antisera used in these tests were from the Center for Disease Control (Atlanta), but we also used commercially available group B antiserum (Burroughs Wellcome Co.) to prepare ARC, and these ARC gave satisfactory grouping reactions when a limited number of streptococci were tested (results not shown).

Edwards and Larson (5) identified group A streptococci by adding ARC to paraffin-ringed colonies on blood agar plates and observing agglutination on the media. We attempted to perform the coagglutination procedure in this manner, but found the results more difficult to interpret than if the test was performed on a glass slide. Apparently the type antigens of group B streptococci can mask or cover the group antigen (9, 11) and thereby interfere with coagglutination grouping of whole cells on media or slides. Thus it was generally necessary to prepare a hot-acid extract of the group B streptococci, which apparently removed the type antigens and allowed the coagglutination grouping reaction to occur. The coagglutination grouping of whole streptococcal cells can be performed on a slide, but if no agglutination occurs, the cells must be extracted and the coagglutination procedure repeated. Care must be taken to pick only pure cultures from plates and subculture when necessary.

The coagglutination typing test was even more rapid than the coagglutination grouping test, since no extraction step was generally required. In fact, if a hot-acid extract is routinely used for the coagglutination typing test, erroneous typing results may be obtained. Twenty-three strains that did type correctly with whole cells as compared with the standard Lancefield hot-acid extraction procedure were also extracted with the modified hot-acid extraction technique, and the coagglutination typing test was repeated. When the test was repeated using modified hot-acid extracts, it was found that 44% of the strains were incorrectly typed as compared with the standard Lancefield typing results. A cell extract should only be used for typing if whole streptococcal cells fail to agglutinate the typing ARC. The coagglutination typing test was very reliable, since 98.5% of the coagglutination typing results agreed with types established by the CP test.

Reagent cells must be prepared from a strain of *S. aureus* that produces sufficient protein A to effectively absorb antiserum. We tested several strains of *S. aureus* that apparently produced small amounts of this protein and consequently gave unsatisfactory agglutination reactions. The Cowan I strain of *S. aureus* was finally selected because it produced large amounts of protein A (3). The method of preparing reagent cells can also influence the amount of protein A produced. When the Cowan I strain of *S. aureus* was grown in a CO₂ atmosphere, these cells failed to absorb antisera. Inferior reagent cells may result if formaldehyde is not immediately removed from treated cells by

washing. Excessive heat treatment of reagent cells will cause them to spontaneously agglutinate. Reagent cells are not difficult to prepare, but must be prepared exactly as described for optimal results.

The coagglutination test must include a negative control consisting of unlabeled reagent cells and the unknown streptococci. In our initial experiments with various types of blood agar plates, nonspecific agglutination of ARC and unlabeled reagent cells was sometimes observed. If the procedure was carried out properly and veal infusion blood agar plates were used, nonspecific agglutination was not observed. If the extract supernatant is contaminated with the aggregated cell sediment, the latter can mimic ARC agglutination. When coagglutination test reactions are observed for more than 4 min, nonspecific agglutination is frequently observed. The above problems are rare in the coagglutination tests, but microbiologists must be aware that they can occur.

This simple coagglutination grouping test should be most useful in busy clinical laboratories, which may not have time to serologically group these streptococci by other methods. The coagglutination typing test should be useful for extensive epidemiological and pathogenicity studies on the group B streptococci.

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