New Method of Grouping Beta-Hemolytic Streptococci Directly on Sheep Blood Agar Plates by Coagglutination of Specifically Sensitized Protein A-Containing Staphylococci

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A technique is described that allows the grouping of beta-hemolytic streptococci directly upon the primary colony. This was accomplished by applying a small drop of specifically sensitized protein A-containing Staphylococcus aureus over a colony of streptococci, rocking the plate to allow mixing of the particles with the soluble group-specific polysaccharide, which in the case of beta-hemolytic streptococci was produced in abundance during colony formation, and observing for agglutination of the sensitized particles. Such a simple test for group A beta-hemolytic streptococci should allow accurate identification of group A streptococci in small laboratories, such as in clinics or physicians' offices, as well as in the larger public health and private laboratories.

A coagglutination reagent described by Kronvall (4) provided a rapid slide agglutination method for typing pneumococci. It has recently been shown to be a simple and rapid method for grouping streptococcal extracts (1) and for the identification of gonococcal antigens (2). We have confirmed and extended the use of sensitized Staphylococcus aureus, Cowan I strain, as described by Kronvall (4), to various groups of meningococci, and Haemophilus influenzae, type b. Their method, although simple, still requires colony transfer, and in the case of beta-hemolytic streptococci chemical extraction of the group-specific polysaccharide was made before identification was possible.

Rapid identification of streptococci would be greatly simplified if a "suspected" colony could be readily and simply identified or grouped without transfer.

This paper describes a simple and rapid method for identification or grouping beta-hemolytic streptococci groups A, B, C, and G directly from the primary colony by a "spot test" which utilizes specifically sensitized protein A-containing staphylococci. This procedure eliminated the relatively long and tedious purification and extraction procedure that has been the standard for beta-hemolytic streptococci grouping since it was described by Lancefield (5).

MATERIALS AND METHODS

Staphylococcal strain. The preparation of protein A rich S. aureus was that described by Kronvall (4) with the following modifications. S. aureus, Cowan I strain (ATC 12558), was grown overnight in Trypticase soy broth (Difco). The bacteria were washed five times with phosphate-buffered saline (PBS: 0.03 M phosphate, 0.12 M NaCl, pH 7.3, with 0.1% sodium azide added as a preservative). The washed bacteria were then suspended in 0.5% formaldehyde in PBS for 3 h at room temperature. The treated bacteria were again washed three times with PBS and made up to a final concentration of 10% in PBS. This suspension of bacteria was then heated over a hot plate with a magnetic stirrer at 80°C for 1 h. The suspension was again washed three times in PBS and stored in PBS at a final concentration of 10%. The heat treatment removed the foul odor of the formaldehyde-treated suspension.

Anti-streptococcal grouping antisera. Rabbit antisera against streptococci were obtained from either the Center for Disease Control (Atlanta) or Difco (Detroit). Antisera were used to sensitize the staphylococcal suspension without further purification or concentration. When non-specificity occurred between certain beta-hemolytic streptococci groups with some lots of grouping sera, the antisera were absorbed by using 0.1 ml of packed washed streptococcal cells per 0.5 ml of antisera. This was carried out at room temperature for 1 to 4 h. Sensitized staphylococcal cells will be referred to as staph-reagent in this report.

Preparation of sensitized staphylococci. Staphylococci were sensitized by the method described by Kronvall (4). The sensitized staph-reagent was used without washing. Excess washing reduced the sensitivity of the sensitized particles. To aid in the visualization of agglutination, 0.01 ml of a 2% Procion brilliant blue, M-RS (Colab Laboratories, Inc., Chicago Heights, Ill.) was added to each 1 ml of 1% staph-reagent.

Preparing blood agar plates. Blood agar base of the following composition was made and sterilized in 100-ml portions in 250-ml flasks: 27 g of blood agar
base (Difco), 10 g of Todd-Hewitt broth (Difco), and 10 g of agar (Difco) were dissolved in 1 liter of distilled water with the aid of heat. The pH was adjusted to 7.8 with sodium hydroxide and the solution was sterilized by autoclaving at 121 C for 15 min under 15 pounds of pressure. Blood agar plates were made using a final concentration of 5% defibrinated sheep blood (International Scientific Industries, Cary, Ill.). The sheep blood agar plates used in the clinical trials were purchased from Gibco (Madison, Wis.).

Stock streptococci cultures were transferred by the conventional loop transfer method and streaked on the sheep blood agar plate to insure isolated colony development. In the clinical trials, culture swabs taken from the patients' throats were "rolled" over an edge portion of a plate to ensure heavy inoculation. A loop was then used to streak the plate in the conventional manner to obtain isolated colonies. All plates were incubated under reduced oxygen tension for approximately 18 h.

Test. A paper clip was bent to make a circle at one end approximately 12 mm in diameter. It was dipped into heated (56 C) liquid paraffin and touched to the blood agar plate so that a beta-hemolytic colony would be centered in the paraffin circle. A drop of the grouping reagent was transferred to the paraffin circle and the plate was rocked to and fro for from 2 to 5 min. Generally, the reaction became positive within 1 to 3 min. The test area was observed for agglutination every 15 s using a dissecting microscope. It was necessary that the plate be tilted to and fro throughout the observation period.

The following streptococcal strains were used in this preliminary study: group A no. 6634; group B no. 99507; group C no. 99778; and group G no. 99501. These were laboratory strains (from human sources) that had been maintained in the lyophilized state for several years. For this study, they were maintained on sheep blood agar plates or in defibrinated sheep blood stored at -20 C. Transfers were made to fresh sheep blood agar plates as required.

RESULTS

A typical agglutination reaction using group A staph-reagent to differentiate group A beta-hemolytic streptococci from other beta-hemolytic streptococci is shown in Fig. 1. Group A staph-reagent was dropped onto each of the 12 transfers. It can be seen that sample C of row 1, A of row 2, and D of row 3 gave positive

![Fig. 1. A typical agglutination reaction pattern of group A staph-reagent when applied to a colony of group A streptococci. In this case, 3 of the 12 beta-hemolytic streptococci were group A and 9 were other groups of beta-hemolytic streptococci.](image)
agglutination. Verification of the group was made by acid extraction (5) and counterimmunoelectrophoresis (3). The other nine samples were either group B or C. It can be seen that there was no evidence of nonspecific agglutination between the group A staph-reagent and either groups B or C.

To determine if the colony, per se, was essential for the agglutination reaction, two "identical" transfers of a group A beta-hemolytic streptococci were made onto a blood agar plate. After 18 h of incubation, one of the colonies was completely removed by scraping and both transfers were tested for their reaction with group A staph-reagent. The result is shown in Fig. 2. The removal of the colony did not make any visible difference between the reaction of the two transfers. This suggests that during growth and colony formation, a large amount of soluble group-specific polysaccharide diffuses into the surrounding media and can react with the staph-reagent. This has further been demonstrated by making the paraffin ring to the side of the streptococcal colony but including a portion of the hemolytic area, with strong agglutination occurring.

To determine the incubation time required to identify a primary transfer of a streptococcal group, the following method was used. A group A transfer was made onto a blood agar plate in eight different areas. Hourly, thereafter, an area was "spot" grouped to determine the reaction intensity. In five separate trials, 5 to 6 h of incubation after a transfer was made gave consistent positive results within 2 min after adding the group A staph-reagent. Some evidence of agglutination appeared with 2 to 4 h of incubation. This data is illustrated in Fig. 3. The figure clearly shows that the intensity of the agglutination reaction increases as the incubation time was lengthened and as the time of observation for agglutination was extended. We set a 5-min time limit on our observation for agglutination to occur because of the possibility that a drying effect on the overlay could be falsely interpreted as agglutination. However, as the incubation period was extended to 6 h or longer, agglutination began to appear within 1

![Image](https://via.placeholder.com/150)

**Fig. 2.** A demonstration to show the colony, per se, apparently was not essential for the grouping reaction. The "blank" circle was a control to insure that nonspecific agglutination did not occur. All three areas were tested with group A staph-reagent. The central area had the colony removed.
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In several hundred testing trials, nonspecific agglutination due to drying never occurred within a 5-min observation period. It must be emphasized that unless the plate is rocked to and fro during the 2- to 5-min observation time agglutination will not occur. Using these procedures, it was possible to group streptococci directly from a primary throat culture blood agar plate. This is shown in Fig. 4, where group A staph-reagent was applied to an area containing three suspected streptococci colonies which had developed after an 18- to 20-h incubation period. Agglutination occurred within 1 min after adding the reagent. Nonspecific agglutination did not occur with unsensitized staphylococci.

DISCUSSION

Lancefield was the first to find that streptococci could be classified into consistent serological groups. In these procedures, it was possible to group streptococci directly from a primary throat culture blood agar plate. This is shown in Fig. 4, where group A staph-reagent was applied to an area containing three suspected streptococci colonies which had developed after an 18- to 20-h incubation period. Agglutination occurred within 1 min after adding the reagent. Nonspecific agglutination did not occur with unsensitized staphylococci.

FIG. 3. These data indicate the incubation time required for a streptococcal transfer to become groupable by the “spot” test. By 2 h, all of the five trials were groupable within 5 min after adding the group A staph-reagent. However, after 6 h of incubation, they were all groupable within 1 min after adding the reagent.

FIG. 4. A typical “spot” grouping test of an 18- to 20-h throat culture from a clinical study. The area with the three colonies (right) was covered with group A staph-reagent; the area on the left was covered with nonsensitized staphylococci.
cal groups by means of group-specific substances extracted from the streptococci. The tests were made using a capillary precipitin test with the corresponding group-specific antisera. This test has been the standard for specific streptococcal grouping for over 50 years. The fluorescent antibody technique (6) was an attempt to rapidly differentiate group A beta-hemolytic streptococci from other beta-hemolytic streptococcal groups and has been extensively used in public health laboratories and in some of the larger private laboratories. It requires expensive equipment, special "dark" rooms, and highly trained technical personnel to insure reliable results. A simplification of the technique to rapidly differentiate group A beta-hemolytic streptococci using immunochemical methods was recently described (3). It was shown to be a simple method, easily performed, and required ordinary laboratory equipment. In this report, we have further simplified the method for grouping beta-hemolytic streptococci. It was accomplished by a simple "spot" test on the original or transferred streptococcal colony. It was evident from these studies that beta-hemolytic streptococci rapidly produce large amounts of group-specific polysaccharide which diffuses through the medium and was readily available to combine with specifically sensitized staph-reagent.

It is now possible, by using this procedure, to identify streptococci within 18 h after the culture has been made. The simplicity of the method would allow rapid grouping of a large number of beta-hemolytic streptococci with minimal technician effort.

Such immunochemical techniques as described in this report for grouping streptococci, theoretically, should permit the extension of the technique to other infectious bacterial agents that have required extensive morphological and biochemical testing for identification. Our preliminary observations show that the technique is applicable to all groups of meningococci and to H. influenzae, type b.

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LITERATURE CITED

1. Christensen, P., G. Kalmeter, S. Jonson, and G. Kronvall. 1973. A new method for the serological grouping of streptococci with specific antibodies adsorbed to protein A-containing staphylococci. Infect. Immun. 7:881-885.
2. Danielsson, D., and G. Kronvall. 1974. Slide agglutination method for serological identification of Neisseria gonorrhoeae with anti-gonorrheal antibody adsorbed to protein A-containing staphylococci. Appl. Microbiol. 27: 368-374.
3. Edwards, E.A., and G. Larson. 1973. Serological grouping of hemolytic streptococci by counter-immunoelectrophoresis. Appl. Microbiol. 26: 899-903.
4. Kronvall, G. 1973. A rapid slide-agglutination method for typing pneumococci by means of specific antibody adsorbed to protein A-containing staphylococcus. J. Med. Microbiol. 6: 187-190.
5. Lancefield, R. C. 1933. A serological differentiation of human and other groups of hemolytic streptococci. J. Exp. Med. 57: 571-586.
6. Moody, M. D., E. C. Ellis, and E. Updyke. 1958. Staining bacterial smears with fluorescent antibody. IV. Grouping streptococci with fluorescent antibody. J. Bacteriol. 75: 553-560.