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Modified Coagglutination Procedure for the Serological Grouping of Streptococci

JAMES R. CARLSON AND LAURENCE R. McCARTHY*

Parasitology and Laboratory Practice, School of Public Health, University of North Carolina, and Clinical Microbiology Laboratories, North Carolina Memorial Hospital, Chapel Hill, North Carolina 27514

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Cowan I staphylococci coated with antisera to streptococcal groups A, B, C, D, F, and G were used as coagglutination reagents in a modified coagglutination procedure (MCAP). Streptococcal group antigens were extracted with a *Streptomyces albus*-lysozyme enzyme mixture for 30 min at 55°C and centrifuged, and the supernatant was tested by slide coagglutination. Positive coagglutination reactions occurred within 30 s. The cell pellets from overnight broth cultures and colonies taken directly from sheep blood agar plates were tested and compared with the results of the Lancefield capillary precipitin method. Of the 102 strains of broth-grown cells tested, 100 were grouped by the MCAP and the Lancefield capillary precipitin method. The remaining two isolates were serologically identified only by the MCAP. Of the original 102 strains, 97 were tested by MCAP after extraction of five well-isolated colonies from a sheep blood agar plate. When this latter method was used, 95.9% of the strains were correctly identified. Nonspecific reactions were observed only while testing the MCAP with the direct plate assay. These cross-reactions were remedied promptly by either absorption or dilution of the antisera involved. The MCAP was found to be a rapid and reliable technique for the serological grouping of streptococci.

Traditionally, the grouping of streptococci has been performed by a capillary precipitin reaction between extracted antigens from the streptococcal cell envelope and group-specific antisera. The group antigens have been extracted by a variety of methods, which include acid hydrolysis (5, 8), autoclaving (12), and the enzymatic extraction by *Streptomyces albus* enzyme either alone (10) or in combination with lysozyme (15). Alternative serological methods for streptococcal identification have included the fluorescent antibody technique (11), counterimmunoelectrophoresis (3), and coagglutination (2).

We have previously performed the serological identification of streptococci by a capillary precipitin method. Streptococcal extracts were prepared by extracting group antigens with a mixture of *S. albus* enzyme and lysozyme. This extraction method, previously described by Watson et al. (15), has proven to be simple, rapid, and reliable. In an attempt to develop a more rapid and accurate procedure for the serological identification of streptococci, we utilized this extraction method in combination with a coagglutination procedure.

This study presents a comparison of this modified coagglutination procedure (MCAP) and the Lancefield capillary precipitin method (LCP).

MATERIALS AND METHODS

Bacterial strains tested. A total of 102 clinical isolates of streptococci were used for testing. Of these, 97 strains were recovered from clinical specimens submitted to the Clinical Microbiology Laboratories of the North Carolina Memorial Hospital, Chapel Hill. The remaining five isolates were obtained from B. L. Wasilauskas of the Bowman Gray School of Medicine, Winston-Salem, N.C. The 102 strains were comprised of 40 strains of group A streptococci, 25 of group B, 7 of group C, 21 of group D (18 enterococci, 3 *Streptococcus bovis*), 4 of group F, and 5 of group G.

Preparation of coagglutination reagents. Coagglutination reagents were prepared by the method of Edwards and Larson (4). The Cowan I strain of *Staphylococcus aureus* was grown overnight at 35°C in Trypticase soy broth and subsequently harvested by centrifugation at 1,500 × *g* for 30 min. The centrifuged cells were washed five times in phosphate-buffered saline (PBS). The washed cells were then suspended in 0.5% formaldehyde for 3 h at room temperature and again washed three times in PBS. A 10% suspension of the Formalin-treated cells in PBS was then heated to 80°C on a hot plate with a stirring bar for 1 h. The heat-treated suspension was washed three times in PBS, suspended to a final concentration of 10% (vol/vol), and stored at 4°C.

Complete coagglutination reagents were prepared by mixing 1.0 ml of the 10% Formalin- and heat-treated suspension with 0.1 ml of an individual commercial

antiserum prepared against one of the following streptococcal groups: A, B, C, D, F, or G. All antisera were purchased from Burroughs-Wellcome, Research Triangle Park, N.C., except for one lot of group F antiserum which was purchased from Baltimore Biological Laboratory, Cockeysville, Md. After a 3-h room temperature incubation, each antiserum-treated staphylococcal suspension was washed three times in PBS and thereafter diluted in PBS to a final concentration of 1% (vol/vol). This 1% suspension represented the complete coagglutination reagent. Each complete coagglutination reagent was stored at 4°C and was stable at this temperature for at least 4 months.

Test procedures. Initial studies were performed by growing each streptococcal strain statically in 60 ml of Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) at 35°C for 18 h. The broth cultures were divided into two 30-ml volumes which were both centrifuged at 1,500 × *g* for 30 min. The supernatants were discarded, and the streptococcal group antigen was extracted from one cell pellet for serological identification by the method of Lancefield (8). The second cell pellet was tested by the MCAP. In the MCAP the cell pellet was suspended in 0.5 ml of an extraction solution. This extraction solution was prepared by reconstituting *S. albus* filtrate (Difco) with an aqueous solution of 5 mg of lysozyme per ml (Sigma Chemical Co., St. Louis, Mo.). The cell suspension was incubated in the extraction mixture at 55°C for 30 min and then centrifuged at 1,500 × *g* for 30 min. Individual drops of the supernatant were independently mixed with 1 drop of individual complete coagglutination reagent on separate slides and then mixed by rocking. A positive coagglutination reaction was manifested by macroscopic agglutination within 30 s of mixing.

Further evaluation of the coagglutination procedure was performed by growing each bacterial strain overnight at 35°C on a 5% sheep blood agar plate (SBAP). One or more well-isolated colonies then were suspended in 0.2 ml of *S. albus*-lysozyme extraction solution. The subsequent extraction and coagglutination procedures were identical to those described earlier for the MCAP.

RESULTS

Of the 102 strains tested, 100 were grouped by both the MCAP and the LCP (Table 1). Only the MCAP identified the two remaining isolates (one *S. bovis* and one group G streptococcus). Repeat testing with the LCP failed to serologically identify these two isolates. No serological cross-reactions were noted between any cell extract, antiserum, or coagglutination reagent employed in the LCP or MCAP during this first phase of testing.

Because of the apparent sensitivity and specificity of the MCAP, we attempted to modify the MCAP so that bacterial colonies from agar plates could be serologically grouped. Seven streptococcal strains were tested. The seven strains used consisted of one strain of streptococcal groups A, B, C, F, and G and two strains of group D streptococci (one enterococcus and one strain of *S. bovis*). One, two, three, and five well-isolated colonies of each bacterial strain were suspended in 0.2 ml of the *S. albus*-lysozyme extraction mixture. The extracts were tested by the MCAP described earlier. As shown in Table 2, all strains except *S. bovis* were correctly identified by the MCAP when five colonies were employed for testing. It was of interest that the group A streptococcus strain tested was correctly identified when only a single colony was extracted.

After these preliminary studies, 97 of the original 102 strains were tested by using the five-colony procedure. As shown in Table 3, all but 4 of the 97 streptococcal strains were identified when five colonies were tested. One group B streptococcal strain and three strains of group D streptococci (two *S. bovis* and one enterococcus) were not identified by the SBAP procedure.

In this latter phase of testing, two technical problems were encountered that were observed neither when the broth culture procedure was employed nor in the development of the method using bacterial colonies. Extracts prepared from five colonies of two enterococci displayed cross-reactions with the group G coagglutination reagent. This cross-reactivity was eliminated by first diluting the group G antiserum 1:4 before

TABLE 1. *Comparison between the MCAP and the LCP for the serological identification of streptococci grown in Todd-Hewitt broth*

Streptococcal group	No. of strains	No. grouped by MCAP	No. grouped by LCP
A	40	40	40
B	25	25	25
C	7	7	7
D	21	21	20
F	4	4	4
G	5	5	4

TABLE 2. *Sensitivity of the MCAP when performed on extracts of colonies taken directly from SBAP*

Streptococcal group	Reaction with the following no. of colonies extracted in 0.2 ml of the enzyme mixture			
	1	2	3	5
A	+	+	+	+
B	w[a]	w	+	+
C	w	+	+	+
D[b]	−	−	−	+
D[c]	−	−	−	−
F	−	−	−	+
G	−	−	−	+

[a] w, Weak positive reaction after 30 s.
[b] Enterococcus.
[c] *S. bovis*.

TABLE 3. *Serological grouping of streptococci by the MCAP using five well-isolated colonies from SBAP*

Streptococcal group	No. of strains	No. grouped by MCAP
A	39	39
B	25	24
C	6	6
D	19	16
F	4	4
G	4	4

preparing the complete coagglutination reagent. Additionally, coagglutination reagents prepared with two lots of group F antisera (one lot from Burroughs-Wellcome and the other from Baltimore Biological Laboratory) reacted serologically with a component of the *S. albus*-lysozyme mixture. This serological activity was eliminated by incubating 0.5 ml of group F antiserum with 0.5 ml of heat-inactivated (100°C for 30 min) preparation of the extraction mixture for 1 h at room temperature. A complete coagglutination reagent prepared with 0.2 ml of the absorbed mixture rather than 0.1 ml of antiserum was successfully used to identify group F streptococci without evidence of serological activity with the extraction mixture.

DISCUSSION

Since its initial use, the coagglutination method for the grouping of streptococci has been shown to be rapid and reliable in comparison with other serological grouping procedures (1, 6, 14). However, inherent with the slide coagglutination procedure have been the problems of multiple agglutinations and autoagglutination noted by many workers (1, 2, 13). These problems are improved by observing the reaction within a short time period (1 to 4 min) (4) and by trypsin treatment of the test streptococci (1, 2, 14). Further still, autoagglutination has been eliminated by employing the cell-free supernatant from broth cultures (9) and by the use of hot-acid extracts (2, 7).

In an attempt to improve the speed and reliability of the coagglutination procedure, we used the *S. albus*-lysozyme enzyme extraction of both the cell pellets from overnight broth cultures and colonies taken directly from SBAP. The possibility of autoagglutination was eliminated by using cell-free supernatants of the enzyme extract. Nonspecific reactions occurred only when a lower antigen concentration obtained when colonies were taken directly from SBAP were used.

When the cell pellets from 30-ml broth cultures were used, there was complete agreement between the MCAP and the capillary precipitin method, except for two strains that could only be identified by the MCAP. All group D strains, including three *S. bovis* species, were reactive when tested with the MCAP. This is significant in light of a recent caution concerning the weak reactivity to group D strains, especially *S. bovis*, of some commercially available group D antiserum (15).

Edwards and Larson (4) attempted to improve the speed of the coagglutination procedure by testing colonies directly on SBAP. This method has not proved successful in our laboratory. Watson et al. (15) were able to remove several colonies directly from SBAP and extract them with the *S. albus*-lysozyme enzyme mixture for successful precipitin testing. By modifying this approach we were able to test colonies removed directly from SBAP, which eliminated the growth of the isolates in broth. When five colonies were used, there was 95.9% correlation with the LCP. Of the 97 strains tested, only 1 group B streptococcus and 3 group D strains (two *S. bovis*) failed to react.

Previously, extensive pretesting (2) or absorptions (4) of antisera were necessary to eliminate nonspecific cross-reactions. In the present study, however, we found our only major problem in two lots of group F antiserum that reacted nonspecifically with a component of the enzyme mixture. This cross-reactivity was easily removed by absorption. A cross-reaction between two strains of group D and a group G reagent was eliminated when the antiserum used to sensitize the G reagent was diluted 1:4. The use of commercially available antisera with the MCAP has proved quite acceptable. We observed no nonspecific reactions in our first phase of testing (extraction of cell pellets from 30-ml broth cultures). The nonspecific reactions that occurred in the second phase of our study were rectified by simple techniques. Lack of cross-reactivity in our study may indicate that the enzyme mixture has the capacity to degrade cross-reacting antigens which may be responsible for the serological cross-reactions observed by other workers.

The MCAP can be performed in less than 1 h. The *S. albus* filtrate and lysozyme are commercially available, and the coagglutination reagent is inexpensive when prepared by a laboratory.

Our study has shown that the MCAP represents a rapid and reliable procedure for grouping streptococci, whether from overnight cultures or directly from primary isolation media.

LITERATURE CITED

1. **Arvilommi, H.** 1976. Grouping of beta-haemolytic streptococci by using coagglutination, precipitation or bacitracin sensitivity. Acta Pathol. Microbiol. Scand. Sect.

B. **84**:79-84.
2. **Christensen, P., G. Kahlmeter, S. Jonsson, and G. Kronvall.** 1973. New method for the serological grouping of streptococci with specific antibodies absorbed to protein A-containing staphylococci. Infect. Immun. **7**: 881-885.
3. **Edwards, E. A., and G. L. Larson.** 1973. Serological grouping of hemolytic streptococci by counter-immunoelectrophoresis. Appl. Microbiol. **26**:899-903.
4. **Edwards, E. A., and G. L. Larson.** 1974. New method of grouping beta-hemolytic streptococci directly on sheep blood agar plates by coagglutination of specifically sensitized protein A-containing staphylococci. Appl. Microbiol. **28**:972-976.
5. **El Kholy, A., L. W. Wannamaker, and R. M. Krause.** 1974. Simplified extraction procedure for serological grouping of beta-hemolytic streptococci. Appl. Microbiol. **28**:836-839.
6. **Hahn, G., and I. Nyberg.** 1976. Identification of streptococcal groups A, B, C, and G by slide co-agglutination of antibody-sensitized protein A-containing staphylococci. J. Clin. Microbiol. **4**:99-101.
7. **Kirkegaard, M. K., and C. R. Field.** 1977. Rapid slide coagglutination test for identifying and typing group B streptococci. J. Clin. Microbiol. **6**:266-270.
8. **Lancefield, R. C.** 1933. A serological differentiation of human and other groups of hemolytic streptococci. J. Exp. Med. **57**:571-595.
9. **Leland, D. S., R. C. Lachapelle, and F. M. Wlodarski.** 1978. Method for rapid detection of group B streptococci by coagglutination. J. Clin. Microbiol. **7**:323-326.
10. **Maxted, W. R.** 1948. Preparation of streptococcal extracts for Lancefield grouping. Lancet **ii**:255-256.
11. **Moody, M. D., E. C. Ellis, and E. L. Updyke.** 1958. Staining bacterial smears with fluorescent antibody. IV. Grouping streptococci with fluorescent antibody. J. Bacteriol. **75**:553-560.
12. **Rantz, L. A., and E. Randall.** 1955. Use of autoclaved extracts of hemolytic streptococci for serological grouping. Stanford Med. Bull. **13**:290-291.
13. **Rosner, R.** 1977. Laboratory evaluation of a rapid four-hour serological grouping of groups A, B, C, and G beta-streptococci by the Phadebact streptococcus test. J. Clin. Microbiol. **6**:23-26.
14. **Stoner, R. A.** 1978. Bacitracin and coagglutination for grouping of beta-hemolytic streptococci. J. Clin. Microbiol. **7**:463-466.
15. **Watson, B. K., R. C. Moellering, Jr., and L. J. Kunz.** 1975. Identification of streptococci: use of lysozyme and *Streptomyces albus* filtrate in the preparation of extracts for Lancefield grouping. J. Clin. Microbiol. **1**: 274-278.