New Method for the Serological Grouping of Streptococci with Specific Antibodies Adsorbed to Protein A-Containing Staphylococci

POUL CHRISTENSEN, GUNNAR KAHLMETER, SVANTE JONSSON, AND GÖRAN KRONVALL

Department of Medical Microbiology, University of Lund, Lund, Sweden

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A coagglutinating reagent has recently been developed and utilized in capsule typing of pneumococci. The reagent consists of stabilized staphylococci coated with specific antibody via the gamma globulin Fc-protein A reaction thereby orienting the antibody molecules with the antigen-reactive Fab parts outwards. Reagent staphylococci coated with antibodies directed against streptococcal group-specific antigens have been used in the present investigations. Overnight cultures of streptococci were treated with trypsin and then directly tested for coagglutination of selected reagent staphylococci on glass slides. In all, 179 strains of streptococci were analyzed by the method described. The results were compared with grouping by using Lancefield extracts in counter-immunoelectrophoresis and were shown to agree completely. The coagglutination method described proved to be accurate, rapid, and simple.

A coagglutinating reagent of great potential use has recently been developed and exemplified in a rapid capsule typing method for pneumococci (G. Kronvall, J. Med. Microbiol., in press). Reagent particles consist of formaldehyde and heat-treated Cowan I staphylococci which are subsequently coated by means of the Fc-protein A reaction with gamma globulin from rabbit antiserum with specificity towards the antigen in question. Specific antibodies adsorbed in this way to stabilized staphylococci will become oriented with their antigen-combining Fab parts directed outwards. A suspension of such staphylococci will be agglutinated by the corresponding antigen only. The antisera used have to be strictly specific as in any other similar immunological method. Preliminary experiments indicated that not only bacterial surface antigens but also viral antigens as well as soluble antigens could be identified and semiquantitated by use of such reagents (G. Kronvall, in press).

Protein A of Staphylococcus aureus is a unique cell wall-associated protein molecule showing a reactivity of high affinity with the Fc part of immunoglobulin G (IgG) (6, 9, 11). In the human, only IgG-1, -2, and -4 molecules seem to react with protein A, whereas immunoglobulins G-3, A, M, D as well as E are negative (10, 13). Since Fc and pepsin component II do not show any protein A reactivity in contrast to Fc fragments as well as H-chain preparations, the reactive sequences are probably located in the CH₄ domain region. Similar Fc structures seem to be present in serum samples of all mammalian species (12).

The group determination of streptococci is most commonly performed by using various precipitation techniques with group-specific antisera and extracts of streptococcal group antigens (3, 8, 14, 16, 18, 22) and also by immuno-fluorescent methods (7, 17). Direct agglutination of untreated streptococci by group-specific antisera does not work regularly for two reasons: first, group-specific antigens are usually not exposed, and secondly, many strains show saline agglutinability (15, 19). Trypsin treatment of streptococci, however, eliminates most of these drawbacks and does, in fact, permit a grouping by means of agglutination (19; N. Riskaer, Ph.D. thesis, Ejnar Munksgaard, Copenhagen, 1943). Experience with this method is based on years of practice at Statens Serum-institut, Copenhagen, Denmark (E. Kjems and Beate Perch, personal communication).

The new type of serological reagent described previously (G. Kronvall, in press) is, in many respects, superior to precipitation techniques as well as simple bacterial agglutination. It was therefore of interest to try its application in the
grouping of streptococci. A combination of trypsin treatment of streptococci with the use of coagglutinating group-specific reagent staphylococci might provide a convenient grouping method for streptococci. The new technique is accurate, rapid, as well as very simple to perform.

**MATERIALS AND METHODS**

**Streptococcal strains.** Strains of streptococci were obtained from routine cultures analyzed at the Department of Clinical Microbiology, University Hospital, Lund, Sweden. They were isolated from throat swab cultures, urine specimens, vaginal discharges, bronchial secretions, and in one case from eye pus. In all, 143 such strains were studied in the experiments. The strains were grown on blood agar plates aerobically or in Todd-Hewitt broth at 37 C. Nine group A streptococci of various T types were obtained from Ebbe Kjems, Statens Seruminstitut, Copenhagen, Denmark. Twenty-nine strains of groups B, C, D, G, L, and N were kindly supplied by Ernst Thal, Statens Veterinârmedicinska, Anstalt, Stockholm, Sweden.

**Antistreptococcal group-specific antisera.** Rabbit antisera against streptococcal group antigens A, B, C, D, and G were kindly supplied by Ebbe Kjems, Streptococcus Department, Statens Seruminstitut, Copenhagen, Denmark, as well as purchased from Difco Laboratories, Detroit, Mich., and from Wellcome Research Laboratories, Beckenham, England. Preliminary experiments indicated that antiserum against group A, B, and D from Difco and against group C and G from Statens Seruminstitut gave clear-cut coagglutination reactions with the corresponding strains. Some of the other sera showed cross-reactivities. The variability among different commercial and noncommercial antisera has previously been pointed out in immunofluorescence studies by Franke et al. (7) and Pavlova et al. (17).

**Grouping of streptococci by using counter-immunoelectrophoresis.** Group determination of streptococci was performed by using group-specific antisera (Wellcome) in counter-immunoelectrophoresis (T. Wadström, A. A. Lindberg, R. Möllby, and C.-E. Nord, Abst. MM no. 29, Medicinskt Riks-stämma, Stockholm) against group antigen extracts made by the method of Lancefield (15). The method has been employed in routine bacteriology since 1971 at the Department of Clinical Microbiology, University Hospital, Lund, Sweden.

**Trypsin treatment of streptococci.** Trypsin treatment followed Riskaer's modification (N. Riskaer, Ph.D. thesis) of Allison's method described by Rudd et al. (20). Overnight (2 ml) or 4-h cultures (10 ml) of streptococci in Todd-Hewitt broth were centrifuged and the supernatant fluids were discarded. Bacterial pellets were suspended in 0.5 ml of 0.2 M tris(hydroxymethyl)aminomethane (Tris), pH 8.0, and 0.1 ml of a trypsin solution (Sigma), 5 mg per ml in distilled water, was added. The bacteria were incubated at 37 C for 1 h and then tested directly with specific reagent staphylococci. No washing or centrifugation was found necessary, in accordance with the findings of Riskaer (Ph.D. thesis). It was found essential not to overload the proteolytic capacity by adding too many bacteria for trypsinization.

When a streptococcus was present in pure culture on a blood agar plate, a very rapid grouping procedure was possible. The bacteria were removed from the blood agar plate by using a sterile swab, suspended directly in tubes (3) containing trypsin buffer solution, incubated for 1 h at 37 C, and then tested with group-specific reagents.

**Preparation of reagent staphylococci.** The stabilization and coating of staphylococci to make specific coagglutinating reagent followed procedures described previously (G. Kronvall, in press). The Cowan I strain of S. aureus (NCTC no. 8530) was grown overnight in CCY broth (1), and the washed bacteria were treated for 3 h with 0.5% formaldehyde in phosphate-buffered saline (PBS, 0.03 M phosphate, pH 7.3, 0.12 M NaCl). After further washings the bacteria were suspended to 10% in PBS. This suspension was treated with heat by being pumped at a rate of 500 ml per h through a metal tube (182.88 cm long, 4 mm inner diameter) submerged in an 80 C water bath. The bacteria were thereby exposed to 80 C for 4 min and then quickly cooled. After additional washings the bacteria were suspended to 10% in PBS containing 0.1% sodium azide and kept at 4 C until use.

For coating of the protein A-containing, formaldehyde- and heat-stabilized staphylococci, 1 ml of the 10% suspension was added to 0.1 ml of specific rabbit antiserum. After mixing the suspension, the staphylococci were washed twice and suspended to 1% in PBS containing 0.1% sodium azide. The suspension was stable for months when stored at 4 C.

**RESULTS**

**Grouping of streptococci by using coagglutinating reagent staphylococci.** One drop of each of the streptococcal group-specific reagent staphylococci was mixed with one drop of trypsinized streptococci on a glass slide and observed with the naked eye while tilting to and fro gently. In positive cases, coagglutination occurs within 30 s with the corresponding reagent. Typical coagglutination patterns are seen in Fig. 1. The resulting coagglutinations were slightly different in character in the various streptococcal groups, e.g., B coagglutination, which gave a very finely granular agglutinate.

Streptococcal strains (143) were trypsinized and subsequently tested for coagglutination with selected antistreptococcal reagent staphylococci. The results were compared with the grouping of the strains by using a precipitation method (T. Wadström et al., Abst.), as shown in Table 1. There was an absolute correlation between the results obtained with the two methods. No cross-reactions were seen between the various A, B, C, D, and G strains by
SEROLOGICAL GROUPING OF STREPTOCOCCI

FIG. 1. Coagglutination reactions of trypsinized group A, B, C, D, and G streptococci with the corresponding group-specific reagent staphylococci (wells marked accordingly, magnification x1.8). In the well marked Contr. is a trypsinized streptococcus tested with staphylococci coated with normal rabbit gamma globulin. Note the qualitative differences in the coagglutination reactions in the various groups.

### TABLE 1. Serological analysis of streptococci by using selected coagglutinating, group-specific reagent staphylococci

<table>
<thead>
<tr>
<th>Streptococci (group)</th>
<th>No. of strains showing positive coagglutination with selected group-specific staphylococcal reagents</th>
<th>Anti-A</th>
<th>Anti-B</th>
<th>Anti-C</th>
<th>Anti-D</th>
<th>Anti-G</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>33</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>55</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>37</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>Other</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*The streptococci analyzed were grouped by the method of Lancefield by using counter-immunoelectrophoresis.

using these selected specific reagent staphylococci. Ten strains of *S. epidermidis*, 10 strains of *S. aureus*, 5 strains of *Neisseria catarrhalis*, and 7 strains of diphtheroids were trypsinized and tested against group-specific reagent staphylococci in control experiments. No coagglutination was taking place in any case. Five strains of pneumococci were also trypsin treated and tested with coagglutinating reagents specific for group A, B, C, D, and G. A strong, immediate reaction with group C-specific reagent staphylococci was taking place. No reaction was seen with the other group-specific reagent staphylococci. This apparent cross-reaction is subjected to further investigations.

Thirty-six strains of streptococci used as reference strains in other laboratories were grouped by using the new method employing group-specific reagent staphylococci. As is clear from Table 2, an absolute correlation with the actual groups of the strains was found by using our new method. The presence of T antigens in trypsinized group A streptococci did not give rise to cross-reactions with other group-specific reagents. The antisera used for coating therefore do not seem to contain anti-T antibodies directed against T antigens common to some groups of streptococci. It might be possible, however, to use the present method for T typing also (Christensen, Kronvall, Perch, and Kjems, unpublished data).

In some cases group D streptococci showed a tendency towards auto-agglutination. Since reagent staphylococci were unaffected, the spontaneous auto-agglutination was easily differentiated from a real coagglutination. These
strains also agglutinated in saline as well as in a suspension of staphylococci coated with normal rabbit gamma globulin. Auto-agglutination did not occur after the trypsinized group D streptococci had been kept at 20°C for 24 h.

The number of trypsinized streptococci still giving specific coagglutination with reagent staphylococci was also determined. Strains representing group A, B, C, D, and G streptococci were washed two times in PBS and suspended in 0.5 ml of Tris buffer, pH 8.0. The suspensions were diluted in two fold steps and tested for coagglutination with reagent staphylococci (Table 3). The number of bacteria still giving coagglutination in these tests showed a mean value of about 1.65 × 10⁸ organisms per ml. By using our method as described above, the final concentration of streptococci in the suspension was about 10⁷ per ml.

**Agglutination of reagent staphylococci by group antigen extracts.** Since soluble antigens have been shown to agglutinate reagent staphylococci (G. Kronvall, in press), it seemed of interest to test group antigen extracts of streptococci against the corresponding reagents. One streptococcal strain from each of groups A, B, C, D, and G was boiled for 10 min with 0.1 ml of 0.2 N HCl (15). Tris buffer was added to the supernatant fluid after centrifugation for neutralization. Agglutination of reagent staphylococci could be performed with all corresponding group antigen extracts without cross-reactions taking place. The agglutination reactions appeared much more slowly, however, (10-15 min) as compared to the much more rapid and clear-cut coagglutination with the trypsinized streptococci themselves.

## DISCUSSION

The precipitation method of Lancefield for the grouping of streptococci is still in routine use all over the world. New methods for the extraction of group antigens and for the demonstration of the group antigen-specific antibody precipitation reaction have been developed. With the exception of immunofluorescent methods and the agglutination method of Rosendal (19), the main principles of grouping streptococci by using antigen extracts have not been changed. In this paper a new serological technique has been utilized and applied in a rapid method for the grouping of streptococci. As can be seen from Tables 1 and 2, the results of determination of group antigen by counter-immunoelectrophoresis and the results of streptococcal grouping by using reagent staphylococci were totally concordant. The grouping of streptococci by using coagglutinating, group-specific reagent staphylococci constitutes a very rapid and simple method.

Streptococci belonging to group B and D do not possess surface-associated cell wall proteins. Grouping with the aid of reagent staphylococci can therefore be performed without trypsin treatment of such streptococci. Trypsinization does, however, provide a more homogeneous suspension of these streptococci (Beate Perch, and E. Kjems, personal communication).

Contradictory findings exist concerning the cellular location of the group D antigen (4, 5, 21, 23). Type-specific carbohydrates, on the other hand, are clearly exposed on the surface of both

### Table 2. Results of testing a panel of laboratory strains against selected streptococcal group-specific reagent staphylococci

<table>
<thead>
<tr>
<th>Serological group of streptococcal strains tested</th>
<th>No. of strains</th>
<th>Coagglutination test using group-specific reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>A*</td>
<td>9</td>
<td>++</td>
</tr>
<tr>
<td>B*</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>C*</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>D*</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>G*</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>L*</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>N*</td>
<td>2</td>
<td>-</td>
</tr>
</tbody>
</table>

* SF 130, T-type 1; Richard, T-type 3; Symons, T-type 9; Blackmore, T-type 11; SF 42, T-type 12; Glover, T-type 13; SF 40, T-type 27; Hensson-Glossy, T-type 44; B 3264, T-type B 3264.

* Streptococcus agalactiae: S-B 2; S-B 8; S-B 17; S-B 20; S-B 37; S-B 39.

* Streptococcus dysgalactiae: S-C 4; S-C 18; S-C 19; S-C 29; S-C 31. Streptococcus S-C 1.

* Streptococcus S-D 1.

* Streptococcus: S-G 2; S-G 3; S-G 4.

* Streptococcus: S-L 2; S-L 3; S-L 4; S-L 7; S-L 8; S-L 9; S-L 10.

* Streptococcus: S-N 1; S-N 2.

### Table 3. Determination of the number of streptococci still giving coagglutination with group-specific reagent staphylococci

<table>
<thead>
<tr>
<th>Strains (group)</th>
<th>Chamber counts* (bacteria/ml)</th>
<th>Dilutions of streptococcal suspensions tested for coagglutination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1/1</td>
</tr>
<tr>
<td>A</td>
<td>2.6 × 10⁸</td>
<td>++</td>
</tr>
<tr>
<td>B</td>
<td>1.7 × 10⁸</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>5.1 × 10⁸</td>
<td>++</td>
</tr>
<tr>
<td>D</td>
<td>1.6 × 10⁸</td>
<td>+</td>
</tr>
<tr>
<td>G</td>
<td>2.2 × 10⁸</td>
<td>++</td>
</tr>
</tbody>
</table>

* Petroff-Hausser chamber counts.
group D and group B streptococci (4). The selected antisera used to prepare group-specific anti-B and anti-D reagent staphylococci in our investigations might also detect such type-specific antigens, thereby representing polytype reagents as has been discussed by others using fluorescent techniques (7, 17).

The group-specific antisera used in the present investigations were selected from among those not showing cross-reactions when coated on staphylococci and tested against a panel of streptococcal strains representing group A, B, C, D, and G. Most antisera screened could not be used because of such cross-reactions. Antigroup C antisera from Difco gave positive reactions with many group A streptococci. Wellcome antisera, which give clear-cut precipitation reactions with corresponding Lancefield extracts, were of no use in the method described here. The reason for these nonspecific patterns seems to be the persistence of trypsin-resistant, cross-reacting antigens on the streptococci, e.g., T antigens.

A distinguishing feature of the coagglutination method used is the fact that autoagglutinating strains can be analyzed with specific reagent staphylococci. The presence of agglutinated test bacteria in the homogeneous suspension of nonreacting staphylococci contrasts to the very strong coagglutination of all suspended bacteria when the specific reagent reacts with the corresponding bacterial antigens.

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LITERATURE CITED


