Simplified Coagglutination Test for Serological Grouping of Beta-Hemolytic Streptococci

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A new coagglutination kit consisting of a plastic slide with dried antibody-coated staphylococci was evaluated for the grouping of beta-hemolytic streptococci of groups A, B, C, and G. The test was compared with the classical precipitation test, using hot formamide antigen extracts with 224 strains of groups A, B, C, and G streptococci. An agreement of 100% was found between the new coagglutination and the classical precipitation procedures. No false-positive results were obtained with group F and D streptococci; however, group L streptococci reacted with the group A reagent. The test procedure could be shortened by using suspensions of colonies from overnight cultures on blood agar plates in small volumes of Todd-Hewitt broth without further incubation. All 74 strains of groups A, B, C, and G were correctly identified from suspensions in Todd-Hewitt broth; however, suspending the colonies in 0.9% saline or phosphate-buffered saline resulted in lower sensitivities.

The majority of human diseases caused by beta-hemolytic streptococci are attributed to Lancefield group A strains. However, non-group A streptococci, particularly groups B, C, and G, also cause diseases, some of which have reached epidemic proportion (8, 13, 19, 20). Hence, it is important for clinical laboratories to identify the serological groups of beta-hemolytic streptococci and to record changes in their epidemiological patterns. Serological grouping can be performed by extracting the group-specific carbohydrates and producing a precipitate against a group-specific antiserum, for example, in capillary tubes (12) or by the countercurrent immunoelectrophoresis method (6). In addition to the classical method of grouping by precipitation (17), fluorescent-antibody techniques have been developed (2, 27). All of these methods are relatively laborious and require well-trained laboratory personnel. A slide agglutination test with suspensions of complete cells has been described but has not gained wide acceptance (22). In 1973, however, a coagglutination technique for the grouping of streptococci was described by Christensen et al. (4). Reagents for coagglutination can be prepared by coating antibodies to protein A-containing staphylococci (16). Coagglutination is a simple, rapid, and reliable procedure for the grouping of streptococci and is especially suited for small clinical laboratories. Many reports have appeared about coagglutination tests with self-prepared (5, 7, 14, 26) or commercially available reagents (1, 3, 5, 7, 11, 18, 23, 24). However, the shelf life of these reagents is relatively short (3 months), as is indicated by the manufacturers. To further simplify the coagglutination test procedure and to extend its shelf life, a kit was developed by Organon, Inc., Oss, The Netherlands. The kit consists of white plastic slides with four dots of dried reagents for group A, B, C, and G streptococci, respectively. Suspensions of streptococci can be tested directly on these slides. This study presents an evaluation of this kit by comparison with the classical ring precipitation test with hot formamide-extracted antigens.

MATERIALS AND METHODS

Bacterial strains. Strains used in the trial were submitted as hemolytic streptococci to the National Institute of Public Health, Bilthoven, The Netherlands, for further identification or originated from streptococcal strain(s) collections.

Coagglutination reagents. Several procedures were tried for raising high-titered and specific agglutinating antisera against group A, B, C, and G streptococci in rabbits. The procedure that was finally adopted was as follows. Rabbits were selected after their blood was tested for the absence of group-specific agglutinating antibodies. Vaccine strains of group A, B, C, and G streptococci were cultured overnight in Todd-Hewitt broth (THB; Oxoid Ltd., Basingstoke, England) and were inactivated by heat treatment for 30 min at 56°C. Immunization was done according to the method of Lancefield (17). Antibody titers were determined by agglutination. A 100-μl amount of undiluted serum was incubated with 400 μl of undiluted streptococcal vaccine suspension in a round-bottom
Coagglutination test tube. Agglutination was read after 24 h. The last dilution that gave complete agglutination was taken as the titer of the antisera. Rabbits were bled when the titers were >1,600. Antisera were tested for group specificity against a panel of three to five streptococcal strains of groups A, B, C, D, F, and G (Centers for Disease Control, Atlanta, Ga.) and cultured in THB. Antisera were made group specific by absorptions with cross-reacting strains. In each step, 10 mg of wet absorption cells that showed cross-reactivity were incubated with 30 ml of antiserum for 1 h at 37°C. Then the suspension was centrifuged for 10 min at 3,000 × g. After each vacuumation of rabbits, the antisera showed different patterns of cross-reactivity. Thus, for each rabbit serum, a tedious procedure of testing for a specific reaction and subsequent absorption had to be carried out to obtain reagents of sufficient specificity. The group B reagent was made polyvalent by using a pool of antisera against two subtypes of group B streptococci, types Ia and III. Staphylococcus aureus, strain Cowan 1, was cultured in brain heart infusion broth (Difco Laboratories, Detroit, Mich.), after which the cells were inactivated by treatment with 0.5% Formalin for 3 h at room temperature and then by heating at 80°C for 4 min. Antibodies were coated to the staphylococci according to the procedure of Kronvall (16). The reagents were colored with 1% crystal violet, placed into the four wells of the plastic slides, and air dried at 37°C for 16 h. Slides were sealed in a lubricate sachet.

Antigen preparation. i. Recommendations of manufacturer. Bacterial strains were streaked onto a 5% horse blood agar plate and incubated overnight at 37°C. One beta-hemolytic colony was transferred to 12 ml of THB and incubated overnight at 37°C. From this culture, 2 drops were inoculated into 2 ml of THB and onto a blood agar plate. The broth was incubated for 4 h or, in the case of poor growth, for up to 48 h. These suspensions were used in the coagglutination test. The blood agar plate was incubated overnight at 37°C and served as both a purity test and a stock culture.

The remaining portion of the 12-ml broth culture was centrifuged at 15,000 × g for 20 min, and the pellet was used for antigen extraction by the method of Fuller (10). The extracted antigen was identified serologically by the classical ring precipitation test with antisera prepared by the National Institute of Public Health.

ii. Modified procedure. Strains were cultured on sheep blood agar and incubated overnight at 37°C. A wet inoculation needle was used to suspend sufficient beta-hemolytic colonies in 0.25 ml of 0.9% NaCl, phosphate-buffered saline (pH 7.2), or THB to obtain a clearly turbid suspension (about 10^8 colony-forming units per ml). These suspensions were used for the coagglutination test.

Test procedure. For the coagglutination test, 0.05-ml amounts of the 2-ml Todd-Hewitt broth culture or the colony suspension were applied to each of the four wells of the white plastic slides containing the dried reagents. The bacterial suspensions and the dried reagents were mixed thoroughly with four plastic spatulas. The slides were rocked by hand for up to 1 min and observed for agglutination. Agglutination in one of the four wells identified the groups of the strain being tested.

In cases of multiple agglutinations, the first or the strongest agglutination was considered to be positive. Identical agglutinations with all four reagents were considered to be autoagglutinations.

RESULTS

The results of serogrouping 274 strains of beta-hemolytic streptococci by coagglutination were compared with those of the classical ring precipitation test (Table 1). Coagglutination antigens were overnight broth suspensions. All strains showed complete agreement in both methods. Weak cross-reactions in the coagglutination test, easily discernible from the specific reactions, were observed with 5% of the streptococcal strains. No autoagglutination was observed. To accelerate the procedure of antigen preparation, colonies were tested directly from overnight blood agar cultures. Colonies were suspended in THB, phosphate-buffered saline, or physiological saline. All 33 strains were correctly identified from THB suspensions (Table 2). However, the other two suspending media showed lower sensitivities. No cross-reactions were observed.

The number of strains tested from THB suspensions was further extended, and the results were compared with those of the precipitation test.

TABLE 1. Comparison of grouping 274 strains of streptococci by precipitation test and coagglutination kit

<table>
<thead>
<tr>
<th>Streptococcus group</th>
<th>No. of positive reactions by Precipitation test</th>
<th>No. of cultures with no reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>49</td>
<td>49</td>
</tr>
<tr>
<td>B</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>C</td>
<td>48</td>
<td>48</td>
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<tr>
<td>D</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>F</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>G</td>
<td>47</td>
<td>47</td>
</tr>
</tbody>
</table>

TABLE 2. Influence of suspension medium in colony-testing procedure

<table>
<thead>
<tr>
<th>Streptococcus group</th>
<th>No. of strains correctly grouped in:</th>
<th>THB</th>
<th>0.9% NaCl</th>
<th>PBS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>13</td>
<td>13</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>B</td>
<td>14</td>
<td>14</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>G</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*PBS, Phosphate-buffered saline.
test. All 80 strains were correctly identified except for three group L strains that gave false-positive reactions with the group A reagent (Table 3). The stability of the dried reagents was shown to be high; the shelf life of the test was found to be at least 1.5 years if it was kept at 4°C.

**DISCUSSION**

The classical method for the serological grouping of streptococci, as developed by Lancefield (17), is based on the extraction of group-specific carbohydrate antigens which are identified in precipitation tests by means of specific antisera.

Except for modifications in the extraction procedure, the method has been serving as an accepted standard ever since. However, the extraction methods are laborious and time consuming. As an alternative, immunofluorescence grouping was developed and introduced into several laboratories as a routine method, initially only for group A streptococci and later also for other groups, with varying success (2, 27). A far more simple and quicker procedure for grouping became available when Christensen et al. (4) developed a coagglutination test with a reagent of specific antibodies adsorbed to protein A-containing staphylococci. In this procedure, the reagents are mixed on a slide with a suspension of streptococci, and coagglutination may occur within a few minutes.

Finch and Phillips (9) found 100% sensitivity. Hahn and Nyberg (11) obtained similar results. In both cases, streptococci were cultured for 16 h in THB, and testing was done with the Phadebact Streptococcus Test (Pharmacia Diagnostics AB, Uppsala, Sweden). Attempts were made to reduce the total assay time, but sensitivity was found to be lower in a 4-h culture method (18, 21). Arvillommi et al. (1), however, obtained almost 100% sensitivity when streptococci were cultured for only 2.5 h in a small volume of THB.

With the reagent used in this study, identification of 224 strains of group A, B, C, and G streptococci was performed. The results showed 100% agreement with the classical precipitation test. For most of the streptococci, a 4-h culture was sufficient. Because of the low sensitivity for the B reagent in preliminary experiments, it was necessary to apply polyvalent antisera obtained with two subtypes. This resulted in 100% specificity for the B reagent. These findings are in agreement with results obtained with immunofluorescence techniques (2, 21). The use of rabbits which were found to be free of natural anti-streptococcal antibodies resulted in only weak cross-reactions which did not hamper correct identification. Neither group F (24 strains) nor group D (26 strains) showed any reactions with the reagents. Only the three group L strains showed cross-reactions with the group A reagent. However, group L strains are principally found in animal infections and are very seldom seen in humans.

A further shortening of the total assay time was attempted by several investigators by testing colonies on the blood plate itself (7) or by testing a suspension of cells taken directly from the blood plate. Sometimes this procedure failed (1) or was successful only after extraction of the antigen (15). Better results were obtained by Slifkin et al. (24) and de Maneville et al. (5). This colony-testing procedure reported in our study shows a high sensitivity and specificity. The number of colonies needed to obtain a strong agglutination response was much higher than that reported by Slifkin and Pouchet-Melvin (25) for the Phadebact Streptococcus Test.

Furthermore, it was observed that incubation of the cultures on blood agar tended to show increasing autoagglutination with increasing age. Replacement of this self-prepared blood agar medium by a blood agar based on Columbia agar (Oxoid Ltd.) solved this problem (data not shown).

An important observation in this study is the influence of the suspension medium on coagglutination in the colony-testing procedures. This phenomenon may explain the varying degree of success of the new method as summarized above. Since it was also found that certain brands of THB may give pseudoagglutinations, it is important to check the THB for these phenomena.

When the coagglutination test was applied in the colony-testing procedure, a minimal assay time could be obtained, no further extraction or incubation steps were needed, and manipula-

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**Table 3. Grouping of beta-hemolytic streptococci by precipitation test and by coagglutination kit with THB suspension from overnight blood agar cultures**

<table>
<thead>
<tr>
<th>Streptococcus group</th>
<th>No. of positive cultures by:</th>
<th>No. of cultures with no reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Precipitation test</td>
<td>Coagglutination kit</td>
</tr>
<tr>
<td>A</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>B</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>C</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>F</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>G</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>L</td>
<td>3</td>
<td>3*</td>
</tr>
</tbody>
</table>

* Reaction with the group A reagent.
tions were simplified by the availability of the reagent as dried dots on the reaction slide.

This new coagglutination kit (Strepto-sec, Organon Teknika, Turnhout, Belgium) offers the clinician a sensitive and specific test for the identification of group A, B, C, and G streptococci that is easy to perform, minimizes the technician time requirement, and may be used in very modestly equipped laboratories.

LITERATURE CITED