Direct-Plate Serological Grouping of Beta-Hemolytic Streptococci from Primary Isolation Plates with the Phadebact Streptococcus Test

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The grouping of beta-hemolytic streptococcal isolates by a new direct-plate procedure employing Phadebact Streptococcus Test reagents was compared with the results obtained with the 4- and 24-h Phadebact grouping procedure and with the Lancefield grouping obtained with a capillary precipitin test. The new procedure employed a modification of the Phadebact procedure that permitted the grouping of streptococci on glass slides with a minimum of five primary isolated colonies. When only five to eight colonies were available for direct testing with each Phadebact reagent, coagglutination was better manifested when the colonies were disaggregated on a glass slide in a loopful of Tween 80 solution. Further enhancement of the coagglutination reaction was effected when the respective Phadebact reagents were employed in relatively small volumes. The direct-plate procedure permitted the correct identification of 127 out of 129 beta-hemolytic isolates. The 4-h method correctly identified 192 of the 200 streptococci tested. All of the 200 isolates tested by the 24-h procedure and the Lancefield grouping were correctly identified. The direct-plate Phadebact procedure affords the clinical microbiologist a rapid and reliable means of identifying groups A, B, C, and G beta-hemolytic streptococci. When sufficient numbers of primary colonies are not available for the direct procedure, the 4- or 24-h procedures may be employed.

There are at present many reports that describe a slide coagglutination procedure for the serogrouping of beta-hemolytic streptococci (1, 3, 6, 10-13, 16, 22). This technique employs a reagent that comprises staphylococci coated with group-specific antibody against beta-hemolytic streptococci. The Fc area of the group-specific antibody is attached to formaldehyde and heat-treated staphylococci, whereas the antigen-combining area, Fab, is exposed for immunological reaction (1, 3, 11). Serogrouping by coagglutination is reported to be accurate, reliable, and relatively uncomplicated to perform (6, 10, 12, 16, 22). A coagglutination procedure for grouping streptococci of groups A, B, C, and G is presently commercially available as the Phadebact Streptococcus Test (Pharmacia Diagnostics, Piscataway, N.J.). Recently, the coagglutination procedure was modified to permit the grouping and typing of group B streptococci directly from the subcultured colonies derived from clinical isolates (13).

This investigation concerns the development of a Phadebact procedure that permits the grouping of beta-hemolytic streptococci directly

from primary isolation plates. This procedure was compared with the 4- (22) and 24-h Phadebact methods and with the Lancefield grouping obtained with a precipitin test.

MATERIALS AND METHODS

Organisms. The cultures of bacteria were obtained from clinical specimens and stock cultures. Stock cultures of serogrouped beta-hemolytic streptococci were maintained in Trypticase soy broth containing 15% (vol/vol) glycerol at -70°C (15). Clinical specimens received on transport swabs (culturette; Marion Scientific Corp., Rockford, Ill.) and the stock cultures were streaked on Columbia sheep blood agar plates (Baltimore Biological Laboratories, Cockeysville, Md.) to obtain isolated organisms. The plates were incubated at 35°C under anaerobic conditions in GasPak (BBL) units for 12 to 18 h. The plates were then observed for the presence of beta-hemolytic streptococci with the application of Gram staining and the determination of hemolytic and catalase activity (7, 8, 24).

Isolated colonies of beta-hemolytic streptococci from each clinical primary plate and stock culture were selected and employed for the direct-plate coagglutination method with the Phadebact reagents. One isolated colony from each clinical primary plate and

stock culture was also selected and inoculated into 2 ml of Todd-Hewitt broth (BBL) for use in the 4- and 24-h Phadebact methods. Similarly, one isolate was inoculated into 40 ml of Todd-Hewitt broth for use in the Lancefield grouping by precipitin testing.

Two hundred primary isolates of beta-streptococci were derived from the following locations (numbers of isolates in parentheses): upper (50) and lower (28) respiratory tracts, blood (26), wounds (55), urine (40), and the umbilicus (1). These 12- to 18-h primary isolates were serogrouped by all three Phadebact methods, when possible. The results were compared with those obtained by the autoclave-extraction method.

Reagents. The Phadebact Streptococcus Test reagents containing lyophilized group A, B, C, and G reagents were supplied by Pharmacia Diagnostics. The reagents were reconstituted as suggested by the manufacturer.

Coagglutination procedures. (i) **Direct-plate method.** To first determine the minimum number of beta-hemolytic colonies required to produce a visible coagglutination response, various numbers of colonies of serogrouped stock strains were picked from the blood agar plates with a sterile inoculating needle and placed into a drop of each Phadebact reagent on a ringed slide. Colonies were disaggregated in the reagent by being mixed with the needle for about 10 s. In some experiments, small numbers of beta-hemolytic colonies were first disaggregated on slides containing either a loopful (0.05 ml) of Phadebact buffer or aqueous 0.01% Tween 80 before the Phadebact reagent was added. In other experiments, the amount of Phadebact reagent added to the bacterial suspensions was controlled with the use of a 25- μ l sample-applicating syringe (Bio-Phore; Bio-Rad Laboratories, Richmond, Calif.). The slide was then rocked for 1 min and examined for coagglutination with transillumination against a dark background.

(ii) **Twenty-four-hour procedures.** The Phadebact 24-h method was performed according to the manufacturer's protocol. One drop of each reagent was placed on a ringed-glass slide, and 1 drop of the 24-h test culture was added to each drop of reagent. After the solution was mixed well with applicator sticks, the slide was rotated for 1 min. The drops were then examined for coagglutination.

(iii) **Four-hour procedure.** The Phadebact 4-h procedure was performed as previously described (22). Six drops of a 4-h cell suspension were delivered with a sterile Pasteur pipette and employed to serogroup the organisms with the Phadebact reagents as described above.

(iv) **Lancefield grouping.** Extracts were prepared for the Lancefield grouping by the autoclave-extraction method (19, 20). Commercial antisera (Burroughs Wellcome) for groups A, B, C, D, F, and G were employed in the performance of the precipitin test in capillary tubes (23). All the grouping methods were performed blind relative to each other.

RESULTS

The numbers of beta-hemolytic streptococci on the primary isolation plates derived from the

respiratory tract often were too few to permit a direct Phadebact test. In other cases, the beta-hemolytic colonies were juxtaposed with those of other bacterial species, thus making it quite difficult to "fish" out enough organisms for the direct test. In contrast, the beta-hemolytic streptococci derived from other clinical sources (wounds, blood, etc.) were generally found in relatively large numbers and were distributed on the surface of the isolation plate, facilitating their removal with an inoculating needle without the contamination of other bacterial colonies.

When a loopful of each serological group of beta-hemolytic streptococci employed in this investigation was mixed directly with specific Phadebact reagent, coagglutination was manifest after several seconds, indicating the potential of a direct coagglutination procedure. Coagglutination was not evident when four or fewer colonies of beta-hemolytic streptococci were picked and mixed into each of the specific serological reagents. However, positive serological reactions occurred between 80 and 100 s with five to six colonies.

The effects of variation in colony number and volume of Phadebact reagent were next examined. The volumes of reagent placed on the ringed-microscope slides were 5, 10, 15, or 20 μ l. Four, five, or ten colonies were each mixed with these respective reagent volumes, and observations for coagglutination were made after 1 min. The use of 10 colonies permitted serogrouping with any of the volumes employed. However, when five colonies were employed, 5 to 15 μ l of reagent was required. These results are summarized in Table 1.

Generally, streptococcal colonies derived from serogroup B strains were easily emulsified into specific Phadebact reagents by being mixed with an inoculating needle. On the other hand, those strains belonging to groups A and C were often difficult to disaggregate into these reagents. Better disaggregation of five to eight colonies was required. Thus, the colonies were first mixed into a loopful of Phadebact buffer or 0.01% Tween 80 on a ringed slide. Ten microliters of

TABLE 1. *Relation of coagglutination response to number of beta-hemolytic streptococci colonies and volume of Phadebact reagent*

No. of colonies	Volume of Phadebact reagent (μ l)			
	5	10	15	20
4	— ^a	—	—	—
5	+	+	+	—
10	+	+	+	+

^a — or +, Negative or positive coagglutination response.

specific Phadebact reagents was then added and mixed with an inoculating needle for approximately 10 s and examined after 1 min. Although the reactions of coagglutination were apparent with prior disaggregation of colonies in the buffer, coagglutination was more discernible with prior emulsification in Tween 80.

In lieu of employing a microsyringe to deliver 10 μ l of specific reagent, we permitted small volumes of reagent to drop from the respective Phadebact reagent bottles. These drops, measured with a micropipette, contained approximately 15 μ l. Thus, in subsequent experiments involving five to eight colonies, both Tween 80 solution and relatively small drops of specific Phadebact reagent were employed to make the coagglutination response visible. Since eight or more colonies yielded specific coagglutination that was quite discernible when mixed with the specific Phadebact reagent, the use of Tween 80 and small volumes of reagent was not continued for experiments involving eight or more colonies.

The direct Phadebact test was performed on 129 primary isolates derived from 129 specimens. This direct procedure, when compared with the precipitin method, permitted the correct identification of 127 primary beta-hemolytic isolates from the available 129 isolates obtained from isolation plates containing a minimum of five colonies required for testing with the four Phadebact reagents. These two discrepancies were associated with two organisms. One was a group F organism identified as group A and the other was a group G organism identified as group A. The discrepancies with these two isolates were eliminated with the 4- and 24-h Phadebact methods.

Misidentification of the streptococcal groups occurred with eight isolates when the 4-h reading procedure was performed. Three group A isolates were not agglutinated by any of the Phadebact reagents, and two group C isolates cross-reacted with group G reagent. Three of the Phadebact-nongroupable isolates were group D

organisms that cross-reacted with the four Phadebact reagents.

The 24-h Phadebact readings yielded results that corresponded to those obtained by the extraction method. These results are presented in Table 2.

DISCUSSION

At present, many reports describe methods available for the rapid recognition of beta-hemolytic streptococci. A variety of physiological tests may be employed in the presumptive identification (7, 8) for the serogrouping of these bacteria. Generally, these tests are performed after 18 h of incubation or more. However, the CAMP test may be prepared to yield identification of group B streptococci 5 to 6 h post-inoculation (4).

The recommended approach to the identification of these streptococci concerns serological grouping with specific grouping antiserum (7, 9, 18). Thus, streptococcal groups are specifically recognized according to their antigenic moiety, as described by Lancefield (14). Although the capillary precipitin test is generally employed for this serological identification, the counter-immunoelectrophoresis procedure with a 4-h culture (17) also appears to be applicable (5). Generally, the cultures for these serological procedures are incubated overnight.

The present investigation has introduced a unique modification of the Phadebact test, which, in contrast to other serological procedures, permits the rapid and accurate serogrouping of the majority of the beta-hemolytic streptococci obtained directly from primary isolation plates. Only two strains tested did not correlate with their actual Lancefield grouping. These strains, however, were correctly identified with either the 4- or 24-h reading procedure with the Phadebact test. At present, the direct Phadebact procedure requires at least five colonies per each Phadebact reagent and at least one other for application of the 4- or 24-h reading, when re-

TABLE 2. Comparison among three Phadebact procedures and autoclave-extraction for serogrouping of beta-hemolytic streptococci from primary isolation plates

Group	Phadebact procedures						No. grouped by Lancefield procedure ^a
	Direct		4-h		24-h		
	No. tested	No. correctly grouped	No. tested	No. correctly grouped	No. tested	No. correctly grouped	
A	53	53	72	69	72	72	72
B	66	66	98	98	98	98	98
C	3	3	11	9	12	12	12
G	3	2	10	10	10	10	10
Nongrouped	4	3	8	5	8	8	8

^a Rantz-Randal autoclave-extraction method.

quired. It was observed that some strains of alpha or gamma streptococci may contain cross-reacting antigens with the beta-hemolytic streptococci (8, 9). In keeping with these observations, primary streptococcal colonies should not be picked with an inoculating needle if they are in direct contact with colonies of other genera. In this investigation, only beta-hemolytic streptococci that could be obtained without contamination from other bacteria were employed. Possibly minute alpha or gamma streptococci were inadvertently picked along with the beta-hemolytic colonies. However, the relative predominance of the beta-hemolytic streptococci collected may have antigenically overshadowed any existing cross-reacting organisms.

Although group A streptococci have previously been identified by coagglutination of the bacteria directly on blood agar plates (6), this procedure was recently reported to be more difficult to interpret than that performed on glass slides (13).

The investigation of Kirkegaard and Field (13) demonstrated that group B streptococci can be grouped and typed by coagglutination on glass slides directly from colonies of subcultured isolates. Generally, the group B streptococci in their investigation could be grouped only if a hot-acid extract of the colonies was employed. This extraction procedure, however, was not required for typing the organisms directly from the plates with the new direct-plate grouping system in this study. In this investigation, all the group B organisms were correctly grouped by the direct-plate Phadebact procedure without the requirement of an extraction method. Possibly, the differences in these two observations with regard to the requirement of an extraction step are related to differences in the preparations of the respective reagents. No unusual difficulty in grouping of B streptococci was reported by other investigators employing the Phadebact Streptococcus Test reagents (1, 10-12, 22).

Our data have shown that when relatively small numbers of colonies (i.e., five to eight) are employed per reagent in the direct-plate Phadebact test, better coagglutination is observed if Tween 80 is incorporated in the reactants. Tween 80 is employed, in part, to produce better visual responses with some *in vitro* antigen-antibody tests (21, 25). It was also apparent that specific coagglutination was better effected when the volume of antibody, that is, the respective Phadebact reagent, was relatively small (i.e., about 10 μ l). This enhancement effect may be related, in part, to an antigen-antibody ratio required for visualization in the secondary stage of immunological response (2) for coagglutination. However, a practical aspect of this obser-

vation is that relatively small drops of reagents may be readily obtained with the Phadebact dropping vials, precluding the use of a micro-applicating syringe.

The Phadebact test systems afford the clinical microbiologist a rapid and reliable means to acquire the serogrouping of groups A, B, C, and G beta-hemolytic streptococci. When sufficient numbers of primary colonies are not available for a direct-plate test, the 4-h test may also be easily performed. In those instances when it is not possible to employ either the direct-plate or 4-h reading procedures, the Phadebact 24-h test may be employed with an overnight culture.

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