

Presumptive Identification of Group D Streptococci: the Bile-Esculin Test

RICHARD R. FACKLAM AND MAX D. MOODY

Streptococcal Bacteriology Unit, National Communicable Disease Center, Atlanta, Georgia 30333

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Six tests commonly used for the presumptive identification of group D streptococci were evaluated. Strains tested included 282 group D streptococci and 366 non-group D. Ratios of percentages of group D to non-group D strains which gave positive reactions for each test are as follows: bile-esculin, 100:2; salt tolerance, 88:24; heat tolerance, 100:80; SF broth, 86:1; KF broth, 99:40; and methylene blue milk reduction, 90:17. These data indicate that the bile-esculin test provided a reliable means of identifying group D streptococci and differentiating them from non-group D streptococci. Methodology for reading and interpreting positive reactions and time of incubation of the bile-esculin medium was defined. Evidence of the need for standardization of salt and heat-tolerance tests was obtained.

The recognition and differentiation of group D streptococci have been difficult problems for diagnostic laboratories for many years. The difficulty of obtaining specific, potent group D antisera has caused many laboratories to rely on presumptive physiological and biochemical tests in an attempt to differentiate group D from non-group D streptococci. These tests are primarily based on the criteria of Sherman (16) which were designed to differentiate "enterococcal" streptococci from "viridans" streptococci.

Hall (*unpublished data*), in a recent survey of 90 private and public health laboratories in the United States, found that the laboratories were using one or more of the following tests to distinguish between group D and non-group D streptococci. Seven were using bile-esculin medium (BEM) reaction; 39, salt tolerance; 8, heat tolerance; 16, *Streptococcus faecalis* (SF) broth; and 17, methylene blue reduction in milk. Thirty-three laboratories were performing serological grouping.

Sherman and his co-workers (15-20, 25) showed that the heat-tolerance, salt-tolerance, and 0.1% methylene blue milk (MBM) tests could be used to distinguish between enterococcal and nonenterococcal streptococci. They showed that the enterococci tolerated 60 C for 30 min, grew in the presence of 6.5% NaCl, and reduced MBM, whereas other kinds of streptococci would not.

The methodology of the heat test has not been standardized. Some laboratories heat a sample of a broth culture to 60 C for 30 min, and then streak the culture on a blood-agar plate. Other

laboratories inoculate a fresh tube of medium, and then heat to 60 C for 30 min. Still other laboratories use 62.5 C instead of 60 C. The size of the inoculum used for the test has not been well defined. Neither the medium in which the culture is grown nor the medium in which the test is conducted has been standardized. Furthermore, the medium and size of inoculum for the salt-tolerance test have not been standardized.

Hajna and Perry (6) described SF broth. They reported that presence of *S. faecalis* was indicated if growth and an acid reaction occurred when the medium was incubated at 45 C. SF broth contains an inhibitor, an indicator, and glucose as a fermentable carbohydrate for testing water for fecal contamination. Nonpathogenic group N streptococci were inhibited during incubation at 45 C.

Kenner et al. (9) described KF streptococcal medium for providing presumptive evidence of fecal contamination in water. The medium contains sodium azide as an inhibitor, an indicator, and maltose and lactose as fermentable carbohydrates. They reported that all of the group D streptococci and some members of the viridans streptococci grew and gave an acid reaction during incubation at 45 C. Although KF medium was not designed to provide presumptive evidence for presence of group D streptococci, we included it in our study.

Rochaix (14) called attention to the value of esculin hydrolysis in the identification of enterococci. The enterococci were able to split esculin, but other streptococci could not. Meyer and Schonfeld (10) incorporated bile into the esculin

medium and showed that 61 of 62 enterococci were able to grow and split esculin, whereas other streptococci could not. Weatherall and Dible (24) obtained nearly the same results with similar media. These results received little attention by Sherman (16). Swan (22) used an esculin medium containing 40% bile salt. He reported that a positive reaction on the BEM correlated with a serological group D precipitin reaction. He tested a limited number of strains of streptococci other than group D and stated that the medium needed to be tested with a large number of clinical specimens. Recently, other investigators (9; R. M. Cole, *unpublished data*) have reported high correlation between serological group D reactions and a positive BEM reaction. Facklam and Moody (Bacteriol. Proc., p. 72, 1969) demonstrated that BEM reactions were superior to salt and heat-tolerance tests used individually or in combination to differentiate group D streptococci from non-group D streptococci.

The purpose of this investigation is to give the clinical bacteriologist data derived from a comparison of diagnostic tests. This data is evidence for using a single, rapid, reliable diagnostic test for differentiating group D streptococci from other streptococci.

MATERIALS AND METHODS

Cultures. A total of 925 strains of streptococci were tested. These included 201 stock strains representing streptococci of serological groups A through U and viridans streptococci *S. uberis*, *S. mutans*, *S. salivarius*, *S. sanguis*, and *S. mitis*. These strains were taken from the culture collection of the National Streptococcal Disease Center. In addition, 389 diagnostic strains of group D and alpha and nonhemolytic non-group D strains were received at the Streptococcal Bacteriology Unit of the National Communicable Disease Center (NCDC) between July 1968 and August 1969. An additional 335 clinical strains representing serological groups A through O were selected by their source to insure that the source of specimen had no effect on the BEM reaction. The sources of all diagnostic specimens used in the study are listed in Table 1.

Six stock strains of group Q streptococci were included in the study because reports in the literature had indicated similarity between their carbohydrate antigen and that of group D (12). We were able to demonstrate weak group D precipitin reactions with NCDC group D antisera with extracts of five of the six strains. Their physiological properties, including the BEM reaction, appear to be quite similar to the group D streptococci.

Serological grouping. The serological group of all strains was determined by means of capillary precipitin tests (23). Cultures for Lancefield extracts were first grown in Todd-Hewitt broth (THB, Difco). If extracts failed to react with NCDC grouping antisera

TABLE 1. Sources of diagnostic strains

Source	Group D	Alpha or non-hemolytic, non-groupable	All other streptococci	Total
Blood.....	64	81	42	187
Urine.....	28	14	34	76
Food.....	16	7	6	29
Animal.....	24	28	11	63
Other ^a	58	69	242	369
Total.....	190	199	335	724

^a Abscesses, skin, throat, wounds, or not specified.

A to O, except N, the strains were recultured in THB supplemented with filter-sterilized dextrose (final concentration, 0.1%), and new extracts were prepared. Strains of alpha and nonhemolytic streptococci that failed to react with the grouping antisera after this procedure were considered nongroupable. The demonstration of a group D precipitin reaction was considered paramount for identifying a strain as a group D streptococcus.

Hemolysis. The hemolytic activity was determined by reading pour plates made with either defibrinated rabbit or sheep blood (4.0%) under a broad field microscope. Hemolysis was read from the subsurface colonies (4). Alpha prime, "wide zone alpha," or "incomplete beta" reactions were recorded as alpha-hemolysis. Complete lysis in the proximity of the subsurface colonies was recorded as beta. No lysis was recorded as "none."

Other routine tests included Gram stains performed on 18- to 20-hr broth cultures and catalase activity; the latter was determined by flooding the surface growth of a 24-hr culture on a neopeptone infusion-agar slant with hydrogen peroxide. Release of oxygen was visually observed as an escape of bubbles toward the surface.

Presumptive tests. Media for all tests were contained in 16 by 125-mm screw-cap tubes in 5-ml amounts. Bile-esculin agar was prepared by the method of Swan (22) except that the esculin was autoclaved with the basal medium. Heat tolerance was determined in THB. The inoculated tube was heated to 60 C and held for 30 min, and then cooled under cold tap water. Salt tolerance was determined in Heart Infusion Broth (Difco) with a final concentration of 6.5% NaCl. MBM was prepared by grinding the dye to a fine powder in a mortar and pestle and suspending 1 g of the dye in 100 ml of distilled water. Ninety grams of skim milk was suspended in 900 ml of distilled water and mixed on a magnetic stirrer for 1 hr before the two solutions were combined and sterilized. Single strength concentrations of SF and KF media (Difco) were used in the procedure. By using a Pasteur pipette, all media were inoculated at the same time with two drops of a 24-hr THB culture of the specimen being tested. Media were incubated at 37 C for 3 days, and readings were taken at 24-hr intervals.

Reading the tests. A positive reaction on the BEM is described when one-half or more of the medium is blackened after any time interval. Plus-minus (\pm) reactions were those in which slight to definite blackening of less than one half of the medium occurred. Negative reactions were recorded when no blackening or blackening of less than one half of the medium occurred after 72 hr. Salt and heat-tolerance tests were read by holding the tubes in front of an incandescent light, rotating the tube, and observing for growth. The reduction of MBM was recorded as positive when the methylene blue dye was decolorized. SF and KF broth were read as positive when the indicators revealed an acid reaction had taken place.

RESULTS

Reactions on BEM. All strains of group D and only occasional non-group D streptococci produced some degree of blackening of the BEM. Table 2 shows the BEM reactions of 240 strains of streptococci, representing a wide variety of stock and diagnostic cultures. All of the group D strains were identified correctly at 24, 48, and 72 hr (Table 2); however, 12 (7%) of the non-group D strains were incorrectly identified after 24 hr, 16 (10%) after 48 hr, and 17 (10%) after 72 hr. Data in part B of Table 2 indicate 74 of 76 (97%) group D strains and 163 of 164 (99%) non-group D strains were identified correctly after 24 hr. After 48 and 72 hr, all the group D

strains reacted, whereas three (2%) and five (3%) of the non-group D strains reacted.

These results show that the plus-minus reactions should be considered negative and that the best time for reading the test is 48 hr. In the following experiments, the plus-minus BEM reactions were recorded as negative. Reactions were read at the 72-hr interval for comparison with the other presumptive tests being evaluated.

Comparison of BEM reactions with other common "presumptive" tests. Table 3 shows the

TABLE 2. Reactions of 240 test strains of streptococci on bile-esculin media

Part ^a	Period of incubation	Groups D and Q streptococcal strains		Other streptococcal strains	
		Positive	Negative	Positive	Negative
A	hr				
	24	76	0	12	152
	48	76	0	16	148
	72	76	0	17	147
B	24	74	2	1	163
	48	76	0	3	161
	72	76	0	5	159

^a In part A, all \pm reactions were considered as positive, and in part B as negative.

TABLE 3. Reactions of 648 strains of streptococci in presumptive tests

Reactions in presumptive tests	Stock strains of alpha and groups A to U, except D and Q (167) ^a	Stock strains groups D and Q (92)	Diagnostic strains of alpha and non-hemolytic non-group D (199)	Diagnostic group D strains (190)
BEM +	5	92	5	190
BEM -	162	0	194	0
Per cent BEM +	3	100	3	100
NaCl +	56	72	31	176
NaCl -	111	20	168	14
Per cent NaCl +	34	78	16	93
Heat +	136	92	167	190
Heat -	31	0	32	0
Per cent heat +	81	100	84	100
SF +	0	77	4	167
SF -	167	15	195	23
Per cent SF +	0	84	2	88
KF +	67	91	83	189
KF -	100	1	116	1
Per cent KF +	40	99	42	99
MBM +	38	73	25	181
MBM -	129	19	174	9
Per cent MBM +	23	78	13	95

^a Figures in parentheses represent total number of strains tested for each group.

reactions of 648 strains, representing a wide variety of stock and diagnostic strains, of streptococci in common "presumptive" tests. All tests were read and recorded after incubation at 35 C for 72 hr. Positive reactions on each of the six tests were assumed to represent presumptive identification as group D streptococci and negative reactions to represent presumptive identification as non-group D streptococci.

The BEM reaction identified all group D strains, and only 10 of 366 (2.7%) non-group D strains reacted. The SF broth test failed to give a positive reaction on 38 of 282 (13.5%) group D strains, whereas only 4 of 366 (1.1%) non-group D strains reacted. The four remaining tests showed less satisfactory results; from 13 to 84% of the non-group D strains reacted positively, and from 5 to 12% of the group D strains failed to react positively.

These results show that the BEM reaction is superior to the other presumptive tests evaluated for distinguishing between group D and non-group D streptococci. The high rate of false reactions with the heat and salt-tolerance tests indicates a need for standardization. The KF broth test yielded a high percentage of false reactions with non-group D strains and should not be used to differentiate group D from other kinds of streptococci.

DISCUSSION

BEM reaction. The results obtained show that, when the BEM reaction is interpreted correctly, the test can be a useful diagnostic tool for distinguishing between group D and non-group D streptococci.

Among the strains of diagnostic alpha hemolytic and non-group D streptococci, nine strains showed a plus-minus reaction after 24 hr, and 14 showed the reaction after 48 hr. None of these strains, however, completed the reaction within 72 hr. Six of the 14 strains resembled *S. mutans*; 4, *S. mitis*; 1, *S. sanguis*; and 3 were not identified.

One of the five alpha or nonhemolytic, non-group D strains that gave positive BEM reactions resembled *S. mitis*. The other four were not identified. Two of the unidentified strains resembled group Q streptococci, but identification was not confirmed. The positive reaction with group Q streptococci should not alter the use of the test in the clinical laboratory. Our data show that group Q streptococci should rarely occur in the clinical laboratory concerned with diagnosis of human infections.

Salt tolerance. Several stock strains, but not all, of groups B, C, P, and U streptococci and *S. uberis* tolerated 6.5% NaCl. In most cases,

the growth of strains in 6.5% NaCl broth was slow and was not visible until the 72-hr reading. Growth of group D strains is generally visible after 24 hr. Stock and diagnostic strains of group D streptococci grow well in 6.5% NaCl broth except for strains of *S. bovis* and *S. equinus*. These two group D species are not like the enterococcal group D species in that they do not tolerate 6.5% NaCl. Papavassiliou (13) reported that several strains of *S. faecalis* failed to grow in broth containing 6.5% NaCl but did grow in the salt broth after a second or third transfer on blood-agar. The major difficulty with the salt-tolerance test is in the actual reading of the test. A heavy inoculum may cause over-reading, i.e., reading the inoculum as growth. A lighter inoculum can cause a delay in determining whether the strains tolerate 6.5% NaCl. Another factor that may affect the salt-tolerance test is the medium in which the test is conducted. Enriched media with 6.5% NaCl will probably support growth of many streptococci that would not ordinarily grow in nonenriched media with 6.5% NaCl. All of these facts point to the need to standardize the medium, the size of inoculum, and the length of time the test should be held.

Heat tolerance. The high survival rate of all kinds of streptococci subjected to 60 C for 30 min could be due either to the methodology of the heat test or the medium in which the test was conducted. There are many variations in the methodology of the heat test.

Ayers and Johnson (1) studied the thermal resistance of certain kinds of streptococci to pasteurization. Houston and McCloy (7) and Ayers et al. (2) recognized heat tolerance as a physiological characteristic of enterococci which could be used to differentiate these organisms from other streptococci. These investigators studied the ability of the organism to grow in skim milk after the inoculated milk and inoculum were held at 60 C for 30 min. Dible (5) used the heat-tolerance characteristic of enterococci to isolate these organisms from mixed cultures. He inoculated a broth with a mixed sample, incubated the broth for 24 hr, heated the broth to 60 C for 30 min, added more broth to the culture, and then incubated the culture another day. The organisms were then plated and checked for survival. Bagger (3) modified the technique to study the effect of pH on the heat tolerance of pure streptococcal cultures. His technique consisted of heating an ampoule of culture to 60 C for 30 min, rapidly cooling it, and streaking a loop of the heated broth onto a blood-agar plate. The plate was examined for growth after 24 and 48 hr. By using this method, Bagger could distinguish between enterococci and nonenterococci.

Skadhauge (21), by using the technique of Bagger, demonstrated that these results were not reproducible, i.e., strains that failed to survive the first time did survive on the repeat test. Skadhauge took strains of streptococci that failed to survive 60 C for 30 min and tested them for survival at 52, 54, 56, 58, 60, and 62 C for 30 min. He found that some strains that had been unable to survive 60 C for 30 min were able to survive this temperature, and a few were able to survive 62 C; most were able to survive 58 C on repeat tests. The methodology most widely used is that of inoculating 5 ml of broth from a 24-hr culture and heating the inoculated culture to 60 C for 30 min. The tube is then cooled rapidly, incubated at 35 to 37 C, and periodically observed for growth. Some laboratories use 62.5 C for 30 min; others use a rapid heating method in which the inoculated broth is rapidly heated to 70 C, and then cooled quickly to 60 C for 30 min. The tube is then cooled and incubated at 35 to 37 C and examined periodically for growth.

All of these facts point out that the heat-tolerance test, like the salt-tolerance test, needs to be standardized. Until researchers agree on the methodology of the test and the kind of medium that should be used, we recommend that clinical laboratories discontinue using the heat-tolerance test to establish presumptive identification of group D streptococci.

SF broth. The acid reaction in SF broth for presumptive identification of group D streptococci was a good indicator for the presence of group D streptococci. Only 4 of 366 non-group D and Q strains tested showed an acid reaction in SF broth. However, 70 strains, 50 of which were alpha or nonhemolytic nongroupable strains, grew in the broth but did not show an acid reaction. For this reason, an acid reaction was considered positive, and growth with no acid was considered negative. Some of those strains that grew in the broth at 35 C perhaps would not have grown if the media had been incubated at 45 C, as Hajna and Perry (6) suggested. Not all clinical laboratories have 45 C incubators, however, and we decided to evaluate these tests at the temperature clinical microbiologists most commonly use.

Two-thirds (26 of 38) of the group D strains that were negative in SF broth were either *S. bovis* or *equinus*. The majority of these two group D species failed to grow in SF broth. This characteristic may be useful in distinguishing these group D streptococci from other group D streptococci. This hypothesis needs to be tested with a large number of these species.

KF broth. Our data show that the KF broth test is not a good test for presumptively differentiating

group D streptococci from non-group D streptococci. Many kinds of viridans streptococci gave acid reactions in KF broth. In addition, many streptococcal species grew in KF broth but were unable to give an acid reaction. The final reading was recorded in the same manner as for SF broth; an acid reaction was required for a positive reaction.

KF broth probably is not used and should not be used as a test for recognizing group D streptococci. Many kinds of streptococci can grow and give an acid reaction in the KF broth. This condition limits its usefulness as a differential test.

Methylene blue milk. Sherman and his co-workers (15-20, 25) showed that when the methylene blue concentration in skim milk was increased to 0.1%, the test had some value in its application for differentiating streptococci. They reported that the enterococci and lactic (group N) streptococcal strains could reduce the dye, but all other streptococci were unable to do so. The test is useful in the diagnostic laboratory, because group N streptococci are nonpathogenic and are not found in human infections.

Several, but not all, strains of groups E and G reduced MBM. We had difficulty in preparing MBM. The dehydrated skim milk would not suspend smoothly, and the methylene blue dye had to be ground very finely to get uniform distribution of the dye in the milk. Even after careful preparation, the dye remained unevenly distributed in some tubes of the medium, and this medium could not be used.

If difficulties with preparation of the medium can be overcome, the error may be reduced to acceptable limits of a presumptive test.

These data indicate that the BEM reaction is the test of choice and could be used as a single presumptive test in recognizing group D streptococci. SF broth would be the next best test, followed by MBM and salt-tolerance tests. Heat tolerance and KF broth should not be used as presumptive tests for differentiating group D from non-group D streptococci. A combination of two tests would be the best solution for recognizing group D streptococci. The best possible combination of tests is the BEM test used in conjunction with either salt tolerance or SF broth. A suggestive interpretation of results would be as follows: (i) BEM positive, SF broth, or salt tolerance-positive—enterococcus; (ii) BEM positive, SF broth, or salt tolerance-negative—group D streptococcus, not an enterococcus; (iii) BEM negative, SF broth, or salt tolerance-negative or -positive—non-group D streptococcus.

All data presented in the study were associated with organisms that were pure cultures of gram-positive cocci in chains, catalase-negative, with

the exception of several catalase-positive group D strains, described by Mundt (11).

Other kinds of bacteria may give positive reactions on BEM. We have not tested other bacteria. Often when group D streptococci are mixed with non-group D bacteria, negative or plus-minus reactions may be observed. Therefore, caution must be exercised to insure that the cultures are pure before testing.

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