Presumptive Identification of Group A, B, and D Streptococci on Agar Plate Media

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Several presumptive tests were evaluated for their effectiveness in differentiating streptococci. When the tests were combined into a battery and the resulting reactions were interpreted as patterns, the overall presumptive identification rate was at least 97%. We used the hemolytic reaction, susceptibility to bacitracin and sulfamethoxazole plus trimethoprim (1.25 μ g plus 23.75 μ g), and standard CAMP reactions on sheep blood Trypticase soy agar, and bile-esculin and 6.5% NaCl agar tolerance tests with incubation in candle extinction jars. Subsequently, 98.9% of the group A; 95.3% of the group B; 100% of the beta-hemolytic non-group A, B, or D; 92.3% of group D enterococcal; 100% of the group D non-enterococcal; and 92.8% of the viridans streptococci were presumptively identified. We then used the hemolytic reactions, susceptibility of bacitracin and sulfamethoxazole-plus-trimethoprim disks, CAMP disk reactions on sheep blood Trypticase soy agar and bile-esculin and 6.5% NaCl agar tolerance tests with incubation in normal atmosphere. Subsequently, 98.1% of the group A; 98.6% of the group B; 99.2% of the β hemolytic non-group A, B, or D; 97.5% of the group D enterococcal; 97.6% of the group D non-enterococcal; and 92.4% of the viridans strains were presumptively identified.

Recent advances in the methodology for presumptively identifying group B streptococci (2, 9, 15) have led us to believe that a battery of tests could be designed to improve the presumptive identification of group A, B, D enterococcal, D non-enterococcal, viridans, and beta-hemolytic non-group A, B, or D streptococcal strains. Although we have reported on a battery of tests used in presumptively identifying the above-mentioned categories of streptococci, the tests were a combination of broths, agar slants, and blood agar plates (7). In addition, one test required 48 h of incubation (hippurate hydrolysis). A more convenient method would incorporate all the tests into one or two agar plate procedures and limit the incubation period to 18 to 24 h.

The most persistent source of error in our battery of tests resulted from the susceptibility of beta-hemolytic non-group A, B, or D streptococci to bacitracin. Approximately 8% of these streptococci are susceptible to bacitracin (7, 10). Because these strains are frequently mistaken for group A streptococci, the procedures for identifying these streptococci need to be improved.

This report includes an evaluation of three different agar formulas for the 6.5% NaCl tolerance test, a comparison of results obtained with bile-esculin (BE) and 6.5% NaCl tests in tubes

and plates, and an evaluation of two different batteries of presumptive tests for identifying the streptococci.

MATERIALS AND METHODS

Streptococcal strains. All the streptococcal isolates used in this study were from human specimens submitted to the Streptococcus Laboratory, Center for Disease Control, Atlanta, Ga., by federal, state, and city public health departments during the past 2 years. Body sites from which they were obtained include throat, skin, wounds, blood, and cerebrospinal fluid.

Identification procedures. All isolates were serologically grouped and evaluated for hemolytic and catalase activity according to previously described methods (3). The group D streptococci were characterized by physiological tests described by Facklam (3) and Gross et al. (8). The viridans streptococci were identified in physiological tests described elsewhere (5).

Media and reagents. Trypticase soy agar (BBL Microbiology Systems) plates with 5% washed, defibrinated sheep blood (SB-TSA) were prepared in our laboratory. These plates were used to determine the susceptibility to bacitracin (0.04 U; BBL) and SXT (trimethoprim, 1.25 μ g, plus sulfamethoxazole, 23.75 μ g; BBL) disks. These plates were also used to test the streptococci for CAMP factor in the standard CAMP (2) and the CAMP disk tests (15).

The CAMP disks were prepared as described by Wilkinson (15). Filter paper disks were saturated with concentrated β -lysin, desiccated for 24 h in an evacu-

ation chamber at room temperature, and stored at -20°C . On washed SB-TSA plates, CAMP disks prepared in this manner showed zones of β -lysin activity ranging from 13 to 18 mm in diameter. *Staphylococcus aureus* strain SS-697 was used for the standard CAMP test as well as for preparing the partially purified β -lysin.

Methyl green deoxyribonuclease (DNase) agar (MG-DTA; Difco) was prepared according to the manufacturer's instructions.

Modified BE medium was prepared as previously described (4), except that the medium was poured into 15- by 100-mm petri dishes.

Modified heart infusion 6.5% NaCl agar (HIA-NaCl) was used to test for NaCl tolerance in both batteries of tests. This medium contained the same ingredients as previously described for modified heart infusion broth (4), except that 1.5% agar was added. The agar was poured into 15- by 100-mm plates.

Other NaCl media described by Qadri et al. (brain heart infusion-NaCl; 11) and Abshire (D streptococcus-enterococcus agar; 1) were prepared according to the authors' instructions and poured into 15- by 100-mm petri dishes.

Testing. The inocula for all tests were taken from overnight fresh cultures on SB-TSA. All strains were coded to mask their identity when they were tested. Material obtained in a sweep through the pure culture with an inoculating loop was used to inoculate the washed SB-TSA plates. The inoculum was spread evenly over one-half of the plate, and one streak was made on the other uninoculated half of the plate. For the standard CAMP test, a streak of the β -lysin-producing staphylococcus strain was then made perpendicular to the single streptococcus streak. The two streaks were carefully placed 1 to 2 mm apart. For the CAMP disk test, the disk containing β -lysin was placed 1 to 2 mm from the end of the single streptococcus streak. Bacitracin and SXT disks were placed approximately 50 mm apart on the section of the plate containing the evenly spread inoculum. Another single sweep of inoculum from the overnight culture was used first to inoculate one-quarter of a BE plate and then one-quarter of an HIA-NaCl plate. The inoculum was carefully spread evenly enough not to be visible on the HIA-NaCl plate to ensure that it did not appear as growth after incubation.

The brain heart infusion-NaCl and D streptococcus-enterococcus agars were inoculated in the same manner as the HIA-NaCl agar.

The DNase medium was inoculated with a sweep taken from the fresh overnight culture. A single streak approximately 30 mm long was made on one-fourth of a 15- by 100-mm petri dish containing MG-DTA medium. A stab was made into the agar along the streak.

The agar plates containing the bacitracin, SXT, standard CAMP, BE, HIA-NaCl, and DNase media were incubated in candle extinction jars at 35°C for this first battery of tests.

The second battery of tests, including bacitracin, SXT, and CAMP disks, BE, HIA-NaCl, brain heart infusion-NaCl, and D streptococcus-enterococcus agar, was incubated in a normal atmosphere at 35°C .

All the tests were read after overnight incubation (16 to 24 h). Any zone of inhibition of growth around

the bacitracin and SXT disks was interpreted as a positive reaction. CAMP reactions were considered positive when an arrowhead-shaped area of increased lysis developed at the juncture of the streptococcal and staphylococcal streak in the standard CAMP test and when a crescent-shaped area of increased lysis formed between the streptococcal streak and the β -lysin-containing disk in the CAMP disk test. CAMP reactions were ranked as intermediate when areas of increased lysis developed in shapes other than those described above; these latter reactions were usually considerably smaller than the normal CAMP reactions. The BE reactions were considered positive when there was any blackening of the BE medium. All NaCl tolerance tests were considered positive when any growth was visible, regardless of whether the indicator changed color. The DNase reaction was interpreted as positive when a clear zone developed around the streak or stab growth of streptococci in the MG-DTA medium.

RESULTS

The reactions obtained with 710 strains of streptococci in the first battery of tests are listed in Table 1. Plates containing the test components were incubated in candle extinction jars. Almost all (98.9%) of the group A streptococci were susceptible to bacitracin and resistant to SXT disks. None of the group A strains tested reacted positively in CAMP or BE tests, and none tolerated NaCl media. Intermediate CAMP reactions interpreted as negative were recorded for several group A strains. Almost all group A strains (186 of 190) reacted positively in DNase tests on MG-DTA media.

Only group B streptococci reacted positively in CAMP tests, with 96.7% of 215 strains being positive. Because the CAMP test is more specific than bacitracin, SXT, or DNase procedures, results obtained with the former were interpreted as more important in presumptively identifying the group B streptococci. None of the group B strains tested gave a positive BE reaction or grew on 6.5% NaCl agar.

Most (89.9%) beta-hemolytic non-group A, B, or D strains tested were resistant to bacitracin and susceptible to SXT, and the others were either susceptible (5.6%) or resistant (4.4%) to both bacitracin and SXT disks. All three patterns of reactions were considered presumptive indicators of beta-hemolytic non-group A, B, or D streptococci because these patterns of reactions were more frequently observed with the beta-hemolytic non-group A, B, or D strains than they were with group A or B strains. The beta-hemolytic non-group A, B, or D strains reacted variably on MG-DTA media; 64.2% were DNase positive. Only two of these strains were bacitracin susceptible and DNase positive, whereas the rest reacted negatively in one or

TABLE 1. Reaction patterns of streptococci on seven presumptive tests incubated in an increased CO₂ atmosphere

Streptococci	Test reaction ^a							No.	Presumptive interpretation
	HEM	BAC	SXT	CAMP	MG-DTA	BE	NaCl		
A	β	+	-	-	+	-	-	184	Group A
	β	+	-	-	-	-	-	4	Group A
	β	+	+	-	+	-	-	1	β -not A, B, D ^b
	β	-	-	-	+	-	-	1	β -not A, B, D
B	β	-	-	+	+	-	-	110	Group B
	β	-	-	+	-	-	-	92	Group B
	β	+	-	+	+	-	-	4	Group B
	β	-	+	+	+	-	-	1	Group B
	β	-	-	-	-	-	-	7	β -not A, B, D
C/G/F	β	-	+	-	+	-	-	97	β -not A, B, D
	β	-	+	-	-	-	-	46	β -not A, B, D
	β	-	-	-	+	-	-	3	β -not A, B, D
	β	+	+	-	-	-	-	7	β -not A, B, D
	β	+	+	-	+	-	-	2	β -not A, B, D
	β	-	-	-	-	-	-	4	β -not A, B, D
D enterococcus	$\alpha/\beta/\gamma$	-	-	-	-	+	+	26	D enterococcus
	$\alpha/\beta/\gamma$	-	+	-	+	+	+	8	D enterococcus
	$\alpha/\beta/\gamma$	-	-	-	-	-	+	2	D enterococcus
	α/γ	-	+	-	-	+	-	1	D non-enterococcus
	α/γ	-	+	-	-	+	-	2	D non-enterococcus
D non-enterococcus	α/γ	-	-	-	-	+	-	9	D non-enterococcus
	α/γ	-	+	-	-	+	-	1	D non-enterococcus
Viridans	α/γ	v	+	-	-	-	-	81	Viridans
	α/γ	v	-	-	-	-	-	9	Viridans
	α/γ	v	+	-	-	+	-	7	D non-enterococcus

^a +, Susceptible to bacitracin or SXT disks, positive in CAMP or BE test, and grows on NaCl media; -, resistant to bacitracin or SXT disks, negative in CAMP or BE test, and does not grow on NaCl media; v, variable reactions, with some strains positive and some strains negative. HEM, Hemolysis; BAC, bacitracin.

^b β -not A, B, D is beta-hemolytic non-group A, B, and D.

both tests. However, fewer bacitracin-susceptible strains of beta-hemolytic non-group A, B, or D streptococci than are usually encountered were found. If larger numbers of bacitracin-susceptible group C and G streptococci were found in clinical specimens, the usefulness of the bacitracin test would be more questionable.

All of the enterococcal strains listed in Table 1 reacted positively in BE and NaCl tolerance tests when tested in tubes, but two strains did not react positively in BE tests when tested in plate BE medium, and three did not react positively in NaCl tolerance tests when tested in plate NaCl medium. Even though the enterococci reacted variously to SXT disks, interpreting the SXT reaction in conjunction with results of the NaCl tolerance test led to a more accurate identification. When positive BE and NaCl reactions or negative (resistant) SXT and positive NaCl reactions were obtained, the organisms

were presumptively identified as enterococci. Using these criteria, 36 of 39 (92.3%) enterococci were presumptively identified. The fact that neither criterion was adequate to identify these strains of enterococci prompted us to investigate other NaCl agar formulas. None of the enterococcal strains were susceptible to bacitracin or reacted positively in CAMP tests.

Only 10 strains of non-enterococcal group D streptococci were tested in the battery of tests listed in Table 1. All reacted positively in BE and negatively in NaCl tolerance tests. One strain was susceptible to SXT and none was susceptible to bacitracin or reacted positively in CAMP tests.

Ninety of 97 (92.8%) of the viridans strains were presumptively identified on the basis of negative BE and NaCl tolerance test reactions. The other seven strains (7.2%) were BE positive and NaCl negative and were presumptively iden-

tified as group D non-enterococci. Nine (9.3%) of the viridans strains were resistant to SXT. SXT susceptibility was not used as a differential test for group D streptococci because of the small number of these isolates tested. None of the viridans strains reacted positively in CAMP or DNase tests. Overall, 97.3% of the strains tested in this battery of tests were presumptively identified correctly.

The results of testing three different media to be used in NaCl tolerance tests are shown in Table 2. NaCl tolerance was strongly considered in differentiating the non-beta-hemolytic streptococci and was the most important test in differentiating BE-positive strains. Since all beta-hemolytic group A, B, and non-group A, B, or D streptococcal isolates did not react positively in BE tests, their growth on NaCl agar was not used as a differential criterion. None of the group D non-enterococcal or viridans strains grew on brain heart infusion-NaCl and HIA-NaCl agars, whereas 67 and 85% of the enterococcal strains, respectively, grew on these two media. All but one (97%) of the group D non-enterococcal and 43% of the viridans strains grew on D streptococcus-enterococcus agar (Table 2). Overall, among the three media evaluated, HIA-NaCl agar most clearly differentiated the non-beta-hemolytic streptococci.

The reactions of 765 strains of streptococci evaluated in the second battery of tests are shown in Table 3. None of these strains was included in the first battery of tests. Whereas in the first battery of tests (Table 1) samples were incubated in an increased CO₂ atmosphere, in the second battery samples were incubated in normal atmosphere. The reactions of the group A streptococci to bacitracin and SXT disks were similar to those of the group A strains in the first battery of tests; i.e., 98.7% of the strains were susceptible to bacitracin and resistant to SXT disks. One group A strain reacted positively in the CAMP disk test and was presumptively identified as a group B streptococcus. One group A strain was susceptible and one was resistant to both bacitracin and SXT disks. They were presumptively identified as non-group A, B, or D beta-hemolytic streptococci, because 24.8 and 6.8% of the beta-hemolytic non-group A, B, or D streptococci were susceptible and resistant, respectively, to both disks.

Among the group B streptococci 98.6% of the strains reacted positively in CAMP disk tests. These results are a slight improvement over those with the standard CAMP test (97.2% of the group B strains positive) only because intermediate CAMP reactions were interpreted as positive if the strain was resistant to bacitracin

TABLE 2. Evaluation of three formulas for NaCl tolerance

Streptococci	No. of strains tested	% Positive		
		BHI-NaCl ^a	HIA-NaCl	DSE agar ^b
Group D enterococci	58	67	85	100
Group D non-enterococci	29	0	0	97
Viridans	159	0	0	43

^a BHI-NaCl, Brain heart infusion-NaCl.

^b DSE, D streptococcus-enterococcus.

and SXT disks, and the hemolysis of these strains was typical of group B streptococci. Although two group B strains were resistant to both SXT and bacitracin and were CAMP disk negative, these strains were identified as beta-hemolytic non-group A, B, or D streptococci, because 9 of 133 (6.8%) of the beta-hemolytic non-group A, B, or D streptococci were CAMP disk negative and resistant to SXT and bacitracin.

Most (68%) of the beta-hemolytic non-group A, B, or D streptococci were resistant to bacitracin, susceptible to SXT, CAMP disk negative, and BE negative. However, 25% of these strains were susceptible to bacitracin and SXT and were CAMP disk negative and BE negative. Also, the most common strains that were resistant to bacitracin and SXT, and which reacted negatively in CAMP disk and BE reactions were beta-hemolytic streptococci, non-group A, B, or D, (6.5% of these strains).

Of the enterococcal strains tested, 94% reacted positively as expected in BE and NaCl tolerance tests. The four enterococcal strains that did not react positively in BE tests were presumptively identified as enterococci because they were resistant to SXT and grew in the NaCl tolerance test. This interpretation was possible because only one viridans strain grew in the NaCl tolerance test. Three enterococcal strains that failed to grow in the NaCl tolerance test were presumptively, but erroneously, identified as group D non-enterococcal streptococci. Eighty-one percent of the enterococci were resistant to SXT disks, whereas 84% of the viridans streptococci were susceptible.

Forty-one of 42 group D non-enterococcal strains were presumptively identified correctly in the BE (positive) and NaCl tolerance (negative) tests. All of the group D non-enterococcal strains were *Streptococcus bovis*. The SXT procedure was of no value in differentiating *S. bovis* from other streptococci; i.e., 23 strains were susceptible and 19 strains were resistant to SXT disks. One *S. bovis* strain was presumptively

TABLE 3. Reaction patterns of streptococci on six presumptive tests incubated in normal atmosphere

Identification	Test reactions ^a						No.	Presumptive interpretation
	HEM	BAC	SXT	CAMP	BE	NaCl		
A	β	+	-	-	-	-	155	Group A
	β	+	-	+	-	-	1	Group B
	β	+	+	-	-	-	1	β -not A, B, D ^b
	β	-	-	-	-	-	1	β -not A, B, D
B	β	-	-	+	-	-	131	Group B
	β	+	-	+	-	-	7	Group B
	β	-	+	+	-	-	1	Group B
	β	-	-	-	-	-	2	β -not A, B, D
C/G/F	β	-	+	-	-	-	90	β -not A, B, D
	β	+	+	-	-	-	33	β -not A, B, D
	β	-	-	-	-	-	9	β -not A, B, D
	β	+	-	-	-	-	1	Group A
D enterococci	$\alpha/\beta/\gamma$	-	-	-	+	+	91	D enterococcus
	$\alpha/\beta/\gamma$	-	+	-	+	+	22	D enterococcus
	$\alpha/\beta/\gamma$	-	-	-	-	+	4	D enterococcus
	$\alpha/\beta/\gamma$	-	-	-	+	-	2	D non-enterococcus
	$\alpha/\beta/\gamma$	-	+	-	+	-	1	D non-enterococcus
D non-enterococci	α/γ	-	+	-	+	-	22	D non-enterococcus
	α/γ	-	-	-	+	-	18	D non-enterococcus
	α/γ	+	+	-	+	-	1	D non-enterococcus
	α/γ	+	-	-	-	-	1	Viridans
Viridans	α/γ	v	+	-	-	-	137	Viridans
	α/γ	v	-	-	-	-	21	Viridans
	α/γ	v	-	-	+	-	6	D non-enterococcus
	α/γ	v	+	-	+	-	6	D non-enterococcus
	α/γ	+	-	-	-	+	1	D enterococcus

^a See footnote a, Table 1.^b See footnote b, Table 1.

identified as a viridans streptococcus because it did not react positively in the BE test.

Among the viridans streptococci evaluated with the tests listed in Table 3, 92.4% were presumptively identified correctly by negative reactions in BE and NaCl tolerance tests. Twelve strains were incorrectly identified as group D non-enterococci because they reacted positively in BE tests. One strain (*Streptococcus uberis*) was incorrectly identified as an enterococcus because it grew in the NaCl tolerance test and was resistant to an SXT disk. Sixteen percent of the viridans strains were resistant to SXT disks. The reaction to SXT disks by viridans streptococci was helpful but inconclusive in differentiating the organisms. Overall, 97% of the streptococci were correctly categorized by their reactions to the tests listed in Table 3.

Table 4 shows the percentage of positive BE and NaCl reactions on tubed (2-day) and plate (overnight) media. There was very little difference between BE reactions of the group D enterococcal and those of non-enterococcal strains

on BE plates and in BE tubes. Also there was very little difference between the NaCl broth and NaCl plate reactions of any of the strains. The greatest difference in results obtained with the tube and plate media appeared in the BE reactions of the viridans streptococci. Substantially fewer viridans strains reacted positively in BE tests on BE plates than in those on BE tubes.

Since it was necessary to interpret the intermediate CAMP reactions as negative indicators of group B streptococci, intermediate CAMP reactions of the streptococci obtained with both the standard and disk methods were evaluated (Table 5). The percentage of group A streptococci which reacted intermediately in both types of CAMP tests was high enough to introduce a large error into the results of the battery of tests if the intermediate reactions were interpreted as positive. However, the interpretation of intermediate CAMP reactions did not cause any difficulty because the hemolysis of the group A, C, and G streptococci on the SB-TSA was much

TABLE 4. Percentages of positive BE and 6.5% NaCl tests determined in tubes and plates

Streptococci	No. tested	% Positive			
		Tube		Plates	
		BE	NaCl	BE	NaCl
D enterococci	120	100	100	96.6	97.5
D non-enterococci	42	100		97.6	
Viridans	169	20.7	2.3	7.1	0.6

TABLE 5. Intermediate CAMP reactions

Streptococci	No. showing intermediate reaction/ total tested (%)	
	Standard CAMP test	Disk CAMP test
Group A	48/190 (25.3)	31/158 (19.6)
Group B	7/215 (3.3)	5/141 (3.5)
Beta-hemolytic, not A, B, D	3/159 (1.9)	7/133 (5.3)
D enterococci	0/39	0/120
D non-enterococci	0/10	0/42
Viridans	0/97	0/171

more intense than the hemolysis of the group B streptococci. The uniquely characteristic hemolysis of the group B streptococci, used in conjunction with intermediate CAMP reactions, was interpreted to represent positive presumptive identification of group B streptococci.

DISCUSSION

The use of multiple tests to improve the presumptive identification of streptococci is not a new idea. Wallerstrom (14) described a multiple-test scheme for presumptive identification of group A streptococci. He placed disks containing bacitracin, nucleate, and glucose on lawns of beta-hemolytic streptococci on blood agar plates. All the group A strains tested reacted positively in the "triple test"; i.e., group A strains were inhibited by bacitracin, the hemolysis was stimulated by nucleate, and the hemolysis was inhibited by glucose disks. However, only a few non-group A streptococci were tested, and one of 12 group G strains reacted positively in the triple test. Although there was some indication that group B streptococci could be differentiated by the triple test, only two strains were tested.

We previously described a battery of tests with which to identify six categories of streptococci presumptively: group A, group B, group D enterococcal, group D non-enterococcal, viridans, and beta-hemolytic non-group A, B, or D streptococci (7). Blood agar plates were used in determining hemolysis and bacitracin suscepti-

bility. The hippurate hydrolysis and BE and NaCl tolerance tests were performed in tubes containing the proper media.

Two days of incubation were required before 95% of the streptococci could be accurately differentiated into the six categories. The largest error in this presumptive identification was the misidentification of beta-hemolytic non-group A, B, or D streptococci as group A streptococci. About 19% of these strains were misidentified because they were susceptible to bacitracin. Pollock and Dahlgren (10) warned that if bacitracin was used as the major criterion for presumptive identification of group A streptococci, the potential for misidentification was significant, especially if the specimen was taken from the upper respiratory tract or from a wound. Thus, it appears pertinent that criteria for presumptive identification of the beta-hemolytic streptococci from these sources be improved.

Darling (2) described a series of experiments in which he attempted to standardize the CAMP test for presumptive identification of group B streptococci. He found that the test worked best if the streptococci were inoculated onto Trypticase soy agar containing washed sheep erythrocytes. The test material was incubated overnight in a candle extinction jar or in a normal atmosphere. Under these conditions the standard CAMP reactions could be determined and group B streptococci could be presumptively identified after overnight incubation, as compared to 2 days of incubation required for the hippurate hydrolysis test. Wilkinson modified the standard CAMP test by incorporating the β -lysin of the *S. aureus* into filter paper disks, thus eliminating the need for an actively growing culture for the CAMP reaction (15). Both Darling and Wilkinson reported a 100% accurate identification of group B streptococci by their CAMP tests after 18 h of incubation.

Recently, Gunn reported on the use of SXT and bacitracin disks for presumptive identification of beta-hemolytic group A, group B, and non-group A or B streptococci (9). He found that when the disks were placed on an inoculum of streptococci on SB-TSA, group A streptococci were susceptible to bacitracin and resistant to SXT disks, group B streptococci were resistant to both disks, and beta-hemolytic group C, G, and F streptococci were resistant to bacitracin but susceptible to SXT disks. These reactions were determined after overnight incubation in an atmosphere of 5% CO₂. Several strains of group C, F, and G streptococci and one strain of group A streptococci were susceptible to both disks. Gunn suggested that strains susceptible to both disks should be interpreted as presumptive

group A streptococci to prevent any misidentification of group A streptococci. However, from the results of these studies it is apparent that this pattern of reactions is more indicative of beta-hemolytic streptococci, not group A, B, or D, than group A streptococci. Gunn also suggested that beta-hemolytic streptococci resistant to both bacitracin and SXT disks should be presumptively identified as group B streptococci. However, combining the results from Tables 1 and 3 indicates that among the beta-hemolytic streptococci, 94.6% of the group B and 2.6% of the non-group B strains were resistant to both disks. However, 97.4% of the group B and less than 0.5% of the non-group B strains gave positive CAMP tests. Therefore, the CAMP reaction is more specific than the combined interpretation of the two disks.

It seemed reasonable that if the bacitracin, SXT, and CAMP tests could be performed on one plate, the presumptive identification would not only be more convenient but also more accurate. It would also be more convenient if the BE and 6.5% NaCl media could be incorporated into plates and the incubation time could be shortened.

In the first battery of tests, samples were incubated in candle extinction jars at 35°C because both Darling (2) and Gunn (9) suggested that an atmosphere containing increased CO₂ was beneficial in both the standard CAMP and SXT disk tests. In the second battery of tests, samples were incubated in normal atmosphere at 35°C because it may be more convenient for some laboratories.

Both batteries of tests were read after overnight incubation (16 to 20 h). Although some viridans strains did not grow as well in normal atmospheres, this condition did not interfere with the presumptive identification. The total number of correct presumptive identifications in the two atmospheres was comparable: 97.3% in candle extinction jars and 97.0% in normal atmosphere. We suggest that microbiologists use the bacitracin, SXT, CAMP, BE, and NaCl tolerance tests along with the hemolytic reactions to make the same presumptive identifications described in our previous publication (7). The battery of tests should be interpreted as a whole; i.e., results obtained in individual tests should be evaluated as they relate to those obtained in all tests run. Patterns of reactions will be comparable to those described in Tables 1 and 3. We suggest that the streptococci can be presumptively identified by the following criteria.

(i) Group A streptococci are beta-hemolytic, susceptible to bacitracin, resistant to SXT, CAMP reaction negative, and BE negative.

(ii) Group B streptococci are beta-hemolytic, variably susceptible to bacitracin and SXT (although most strains are resistant to both disks), CAMP reaction positive, and BE negative. Occasional nonhemolytic group B strains will have comparable reaction patterns.

(iii) Group C, F, and G streptococci (beta-hemolytic, not group A, B, or D) will react in three patterns to bacitracin and SXT. All strains are beta-hemolytic and react negatively in CAMP and BE tests. Most strains are resistant to bacitracin and susceptible to SXT. An appreciable number of these strains may be susceptible to both bacitracin and SXT. A few strains will be resistant to both bacitracin and SXT.

(iv) Group D enterococcal streptococci vary in their hemolytic reaction; most strains are nonhemolytic, but alpha- and beta-hemolytic strains are common. All strains are resistant to bacitracin and react negatively in CAMP tests. Three patterns of reactions from the SXT, BE, and NaCl tests are characteristic of enterococci. Most strains will react positively in BE and NaCl tolerance tests and be resistant to SXT. Some strains are susceptible to SXT and react positively in BE and NaCl tests. Some strains are resistant to SXT and tolerant to NaCl (NaCl positive), but fail to give a positive BE reaction.

(v) Group D non-enterococcal streptococci are alpha- or nonhemolytic and are usually resistant to bacitracin. However, occasional strains are sensitive, vary in their reactions to SXT disks, and are BE positive and NaCl intolerant.

(vi) Viridans streptococci are alpha- or nonhemolytic and vary in their reaction to bacitracin and SXT. However, most strains are susceptible in SXT and negative in CAMP, BE, and NaCl tolerance tests.

The results of these studies, using the above interpretation, indicate that at least 97% of the medically important streptococci can be presumptively identified. Streptococci that do not fit into one of the reaction patterns should be checked for purity and retested. The retesting can be performed on plates or in tubes as previously described (7). Strains whose reactions in the presumptive tests remain atypical should be serologically grouped and definitively identified in a reference laboratory.

We suggest that the bacitracin, SXT, and CAMP disks be stored at -20°C. (Placing them in the freezer of a regular refrigerator is satisfactory.) We had some difficulty maintaining the activities of SXT and CAMP disks when they were stored at 4°C for even as short a period as 5 days, although they remain stable for 1 month (with daily use) when stored at -20°C.

Accurate determination of hemolysis is the

first and most useful step in identifying streptococci. Bacteriologists should familiarize themselves with typical hemolytic activities of the streptococci. Although the hemolysis of group A streptococci cannot be routinely differentiated from that of group C or G streptococci, the former differs from that of group B, D, and F streptococci. The zone of hemolysis surrounding group B streptococci is much smaller and the zone of hemolysis surrounding the group D enterococcal streptococci is larger than that of group A. Also, colonies of group D streptococci are much larger than those of other streptococci, and colonies of group F streptococci are much smaller than those of beta-hemolytic group A, B, C, D, and G streptococci. The zone of hemolysis surrounding these small colonies is quite large, nearly equivalent to the zones formed around group A streptococci.

We preferred to read the zones of inhibition of growth around the SXT disks in the same manner as we described for bacitracin (7). Any zone of inhibition of growth around the bacitracin or SXT disks represents a positive result. To require certain zone sizes of growth inhibition, a standard inoculum must be used. However, in tests to obtain a presumptive identification, requiring standardized inoculum would add an unnecessary element of complication.

The CAMP test should be read in conjunction with the hemolytic activity of the streptococci. Beta-hemolytic non-group B streptococci that react intermediately in CAMP tests are easily differentiated from group B streptococci which react weakly or intermediately in CAMP tests because of the differences in hemolytic activity described above. Most group B streptococci are clearly positive in CAMP tests.

The BE plate test is more difficult to interpret than the tube test. Some enterococcal strains react very weakly; i.e., the blackening of the medium may not be apparent unless the plate is inspected in front of a white background. We advise reading the test in this manner and interpreting any blackening of the medium as a positive reaction, even though doing so may result in a number of misidentifications of viridans streptococci as group D non-enterococcal streptococci because of weak BE reactions. This error is medically less dangerous than misidentifying enterococci because antibiotic susceptibilities of the group D non-enterococci are similar to those of the viridans streptococci (13).

One reason that an occasional group D streptococcus was negative in the BE plate test but positive in the BE tube test was the incubation period. The plates were incubated for 18 to 20 h, whereas the tubes were incubated for 40 to 42 h. If the agar plate BE test had been incubated

longer, the reactions might have been more comparable to those obtained with tubed media.

The results with the 6.5% NaCl tolerance tests were the most disappointing of those obtained. We strongly recommend that plates be carefully inspected immediately after inoculation to insure that the inoculum is spread so as not to be read as growth. When the plates are prepared in this manner, any growth interpreted as a positive reaction reflects an accurate indicator NaCl tolerance.

The CAMP disks are not available commercially at present (November 1978). It is hoped that in the future they will be made available or that the media and disks will be commercially available as a kit.

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