Presumptive Identification of Streptococci with a New Test System

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A test is described that could replace bacitracin susceptibility for presumptive identification of group A streptococci as well as 6.5% NaCl agar tolerance for presumptive identification of enterococcal streptococci. The L-pyrrolidonyl-β-naphthylamide test, based on hydrolysis of pyrrolidonyl-β-naphthylamide, was used in conjunction with the CAMP and bile-esculin tests to presumptively identify the streptococci. Among the beta-hemolytic streptococci; 98% of 50 group A, 98% of 46 group B, and 100% of 70 strains that were not group A, B, or D were correctly identified by the new presumptive test scheme. Among the non-beta-hemolytic streptococci; 96% of 74 group D enterococcal, 100% of 30 group D nontenterococcal, and 82% of 112 viridans strains were correctly identified by the new presumptive test scheme.

Bacitracin differential disks have been used for many years to distinguish presumptively between group A and non-group A beta-hemolytic streptococci (7). One potential weakness in the interpretation of the bacitracin result has been lack of specificity. Although nearly all group A streptococci are susceptible to bacitracin (positive presumptive identification), 5 to 20% of the beta-hemolytic non-group A streptococci may be susceptible, resulting in misidentification of them as group A streptococci (3, 8).

Gunn (4) first reported the use of SXT (23.75 μg of sulfamethoxazole and 1.25 μg of trimethoprim) disks with the bacitracin disk to improve the presumptive differentiation of group A from non-group A beta-hemolytic streptococci. We have expanded this presumptive test scheme to include the CAMP, bile-esculin, and 6.5% NaCl tolerance tests with the bacitracin and SXT disks in an effort to further improve the presumptive identification of group A as well as other streptococci (3). Approximately 98% of the group A streptococci tested have been correctly identified by this scheme (3); 6.5% NaCl agar (salt agar) has been used to differentiate between enterococcal and nontenterococcal group D streptococci in the scheme. Sterile paraffin has been added to the surface of the salt agar to prevent the hydroscopic salt quadrant from accumulating moisture during storage. However, a recent report presents evidence that the paraffin wax-coated salt agar does not support the growth of all enterococcal group D streptococci (1). Evidently some of the residual wax on the surface of the salt agar is inhibitory to some enterococcal strains. The failure of enterococci to grow on the salt agar could lead to their misidentification, a potentially serious error because patients with enterococcal infections are managed differently from those with other streptococcal infections.

The test that can replace the bacitracin and 6.5% NaCl tolerance tests uses a agar medium containing L-pyrrolidonyl-β-naphthylamide (PYR) to identify group A and enterococcal streptococci. This test is a modification of the test for L-pyrrolidine hydrolysis described by J. Godsey, R. Schulman, and L. Eriquez (Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, C84, p. 276). The evidence suggests that the agar-based PYR test is more specific than the bacitracin test for group A streptococci and is at least as specific as the 6.5% NaCl tolerance test for enterococci.

MATERIALS AND METHODS

All of the streptococcal isolates used were from human specimens submitted to the Streptococcus Laboratory, Centers for Disease Control, Atlanta, Ga., by federal, state, and city public health departments. Body sites from which they were obtained include, throat, skin, wounds, blood, and cerebrospinal fluid. All strains were identified by conventional serological (Lancefield extracts) and physiological tests (2). The Streptococcus identification plates (CSM Strep-ID plates) were obtained from Carr-Scarborough Microbiologica ls, Inc., Stone Mountain, Ga. The plate is divided into three sections: one contains tryptic soy agar supplemented with 5% washed, defibrinated sheep blood; one contains modified bile-esculin agar (Difco Laboratories, Detroit, Mich.); and one contains PYR agar. The modified bile-esculin agar was prepared by resuspending the powder according to the
manufacturer's instructions and sterilizing the agar but omitting the horse serum additive. The CSM Strep-ID plate was supplied with CAMP disks (9) and PYR reagent.

Two or three colonies were taken from an overnight blood agar plate culture and streaked onto each of the three media with a sterile inoculating loop. The CAMP disk containing staphylococcal beta-lysin was placed 1 to 2 mm from the end of a single streak of the streptococcus culture on the tryptic soy agar (10). The bile-esculin and PYR media were streaked with a back-and-forth motion with the inoculating loop containing the streptococcus culture. The plates were incubated for 16 to 20 h at 35°C in a normal atmosphere.

CAMP reactions were scored positive if a crescent-shaped area of increased hemolysis formed between the test isolate streak and the beta-lysin-containing CAMP disk. CAMP reactions were scored as intermediate if areas of lysis developed in shapes other than a crescent (or arrowhead). Intermediate CAMP reaction areas of lysis are usually smaller than a normal CAMP and are quite distinctive. In our test, intermediate CAMP reaction areas were interpreted together with the hemolytic activity as previously described (3). Bile-esculin reactions were scored positive if there was any blackening of the medium.

The PYR agar was used to test for the enzymatic hydrolysis of l-prolylglutamic acid-β-naphthylamide by streptococcal test isolates. The free β-naphthylamine released upon hydrolysis of the aminopeptidase substrate was detected by the addition of PYR reagent, which was dropped directly upon test plate growth. The formation of a cherry-red color within 2 min was considered a positive test. The development of a yellow or light-orange color was considered to be a negative test. The PYR reagent is an acid solution of N,N-dimethylanilinoenamaldehyde containing detergent that forms a red Schiff base with free β-naphthylamine. In contrast, β-naphthalamides are not reactive since the amino group is involved in the amide bond.

Bacitracin susceptibility testing was performed on the beta-hemolytic strains according to previously described procedures (2).

The presumptive identification of the streptococci is based on the test results listed in Table 1.

**RESULTS**

The presumptive identification of 402 strains of streptococci and aerococci is given in Table 2.

Of the beta-hemolytic streptococci, only two were misidentified by the presumptive test scheme. One each of group A and B streptococci was identified as beta-hemolytic streptococci not of group A, B, or D. The group A streptococcus, in addition to being negative in the PYR test, was bacitracin resistant and did not multiply in the in vitro bactericidal test (6). This indicates that although the strain possesses the group A antigen, it is not like typical group A streptococci (S. pyogenes), which survive and multiply in the bactericidal test because they possess antiphagocytic factors. The other 49 strains of group A streptococci were susceptible to bacitracin and were PYR test positive. One group B, four group C, and five group G strains were susceptible to bacitracin. None of the group F or beta-hemolytic nongroupable strains was susceptible to bacitracin. None of the nongroup A beta-hemolytic strains was PYR test positive. The group B streptococcus that was misidentified did not produce CAMP factor.

Of the enterococci strains (S. faecalis, S. faecium, S. durans, and S. avium), three strains of S. faecium were incorrectly identified. One S. faecium strain was presumptively identified as a group D nonenterococcus because it gave a negative PYR test, and two strains remained unidentified because they gave only a positive PYR test. This reaction pattern (non-beta-hemolytic, CAMP, and bile-esculin negative, PYR positive) is not accounted for in the scheme summarized in Table 1, thus the strains remained unidentified.

All 30 strains of group D nonenterococci (S. bovis biotypes I and II and S. equinus) were correctly identified.

Of 112 viridans streptococci, 20 were misidentified as group D nonenterococci because they gave positive bile-esculin reactions. Among the viridans species, half (16 of 32) of the S. intermedius strains tested were misidentified as group D nonenterococcal streptococci.

All five nonhemolytic group B streptococci were correctly identified.

Three different patterns of reactions resulted from testing 13 strains of aerococci. Six strains

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**TABLE 1. Presumptive identification of streptococci based on hemolytic activity and the reactions on CAMP factor, pyrrolidine, and bile-esculin media**

<table>
<thead>
<tr>
<th>Streptococcal presumptive identification</th>
<th>Hemolysis</th>
<th>Presumptive test CAMP</th>
<th>PYR</th>
<th>Bile-esculin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>Beta</td>
<td>−a + −</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group B</td>
<td>Beta</td>
<td>−a + −</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group D streptococcus, enterococci</td>
<td>Alpha</td>
<td>− + +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group D streptococcus, nonenterococci</td>
<td>Alpha</td>
<td>− + +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viridans</td>
<td>Alpha</td>
<td>− + +</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Some strains gave intermediate CAMP reactions.
Confirmatory identification | No. tested | Group A | Group B | Beta-hemolytic streptococcus not of Group A, B, or D | Group D enterococci | Group D non-enterococci | Viridans
--- | --- | --- | --- | --- | --- | --- | ---
A. Beta-hemolytic
Group A | 50 | 49<sup>a</sup> | - | 1 | - | - | -
Group B | 46 | - | 45 | 1 | - | - | -
Group C | 21 | - | - | 21 | - | - | -
Group D | 8 | - | - | 8 | - | - | -
Group G | 31 | - | - | 31 | - | - | -
No Group | 10 | - | - | 10 | - | - | -
B. Non-beta-hemolytic
*S. faecalis* | 51 | - | - | 51 | - | - | -
*S. faecium<sup>b</sup>* | 16 | - | - | 13 | 1 | - | -
*S. durans* | 2 | - | - | 2 | - | - | -
*S. avium* | 5 | - | - | 5 | - | - | -
*S. bovis I* | 17 | - | - | - | 17 | - | -
*S. bovis II* | 12 | - | - | - | 12 | - | -
*S. equinus* | 1 | - | - | - | 1 | - | -
*S. anginosus* | 6 | - | - | - | - | 6 | -
*S. intermedius* | 32 | - | - | - | 16 | 16 | -
*S. mitis* | 10 | - | - | - | - | 10 | -
*S. morbillorum* | 1 | - | - | - | - | 1 | -
*S. mutans* | 15 | - | - | - | - | 15 | -
*S. salivarius* | 13 | - | - | - | 2 | 11 | -
*S. sanguis I* | 16 | - | - | - | 2 | 14 | -
*S. sanguis II* | 8 | - | - | - | - | 8 | -
*S. uberis* | 4 | - | - | - | - | 4 | -
not identified | 7 | - | - | - | - | 7 | -
Group B | 5 | - | 5 | - | - | - | -
Aerococci<sup>b</sup> | 13 | - | - | 6 | 1 | - | -

<sup>a</sup>- , None.
<sup>b</sup>Two *S. faecium* and six aerococcal strains gave positive PYR tests but negative reactions on all other tests.

were identified as group D enterococci, one was identified as a group D nonenterococcus, and six others remained unidentified because only the PYR reactions were positive.

**DISCUSSION**

The presumptive identification of streptococci has two advantages over confirmatory identification procedures. The cost of the reagents for presumptive testing is generally less than that for confirmatory testing, and presumptive tests are usually less complex to perform than are confirmatory tests. Although both the bacitracin and PYR tests are presumptive tests for identification of group A streptococci, the PYR test is more specific than the bacitracin test because no group C or G beta-hemolytic streptococci were PYR positive, but 14.5% of these same strains were positive by the bacitracin test. The sensitivity of the bacitracin and PYR tests appears equal. True *S. pyogenes* strains are probably susceptible to bacitracin and give positive PYR tests, whereas atypical group A streptococcal strains (non-M-protein-containing group A streptococci) are resistant to bacitracin and give negative PYR tests. We tested several of these atypical group A streptococci in in vitro bactericidal tests, and none of them has survived. This indicates that they do not have the antiphagocytic antigens (M protein) possessed by most, if not all *S. pyogenes* strains. These atypical group A streptococcal strains are more similar to group F strains morphologically and physiologically than they are to typical group A strains (unpublished observations).

CAMP-negative group B streptococci have been previously reported (3, 10). We have reported that 2 of 141 group B strains were CAMP negative (3). In this report, 1 of 46 group B strains tested was CAMP negative. These find-
ings point out the reason why these tests are considered presumptive. Not all streptococcal strains are properly identified by using presumptive tests alone.

The failure of the PYR test to identify accurately all of the enterococci does not mean that it is without value as an aid in the presumptive identification of these organisms. Only 1 of 71 enterococcal strains (1.4%) failed to give a positive PYR test. Using salt tolerance to differentiate bile-esculin-positive strains, we have reported that 3 of 120 enterococcal strains (2.5%) fail to give a positive salt tolerance reaction (3). Although a greater sensitivity with either the PYR or salt agar tolerance tests would be advantageous for identification of enterococci, we have not observed an increase in sensitivity of these tests during our studies. The strains that were negative in the PYR or salt agar tolerance tests grew in 6.5% NaCl heart infusion broth (2). We suggest that the PYR test be used for screening, and for strains that give atypical reactions, the tube test for both bile-esculin and salt tolerance should be used for confirmation. Another alternative is to perform all of the tests described in the American Society for Microbiology manual (2) to complete the serological and physiological identification of the strains.

In this study an appreciable number of viridans streptococci (18%) were positive on bile-esculin agar, and a few enterococci (2.8%) were negative on bile-esculin agar. We have previously reported that 12 of 171 (7%) viridans streptococci were positive on bile-esculin agar and 4 of 120 (3.3%) enterococci were negative on bile-esculin agar (3). Since we selected test strains from recently submitted specimens; the high percentage of bile-esculin-positive viridans strains in the sample is probably due to an increased interest in the identification of S. bovis. An appreciable number of bile-esculin-positive salt tolerance test-negative strains submitted to our laboratory are not S. bovis but S. MG-intermedius. This does not decrease the value of the PYR test for identifying enterococci, but it does point out the potential error in presumptive identification of S. bovis. This is not a serious error because the antimicrobial susceptibilities of the group D nonenterococcal and viridans strains are similar (9), and the antimicrobial treatment of the infections may be the same. However, the misidentification of blood culture isolates as S. bovis may lead to erroneous assumptions concerning a diagnosis of colon cancer (5).

The results with the aerococcal strains point out a fact that we have previously noted: these tests are presumptive, and in some cases erroneous or inconclusive results are observed. The aerococci are not frequently found in human infections, but when they are, erroneous or inconclusive identification or both can result. The reactions of the aerococci on PYR medium are not appreciably different from those that would be obtained with 6.5% NaCl agar. The majority of aerococcal strains are positive on both media. The presumptive identification of aerococci is difficult if not impossible. We can only suggest that if a microbiologist suspects that an unknown strain may be an aerococcus, the tests described in the American Society for Microbiology manual should be used to complete the identification.

If the battery of tests described in Table 1 is used for presumptive identification of streptococci, more than 98% of the beta-hemolytic streptococci and more than 89% of the non-beta-hemolytic streptococci will be correctly identified. The PYR test appears to be more specific than the bacitracin test for identification of group A streptococci and appears to be equal in sensitivity to the bacitracin test. The PYR test appears to be equal to the agar salt tolerance test in the differentiation of enterococcal and nonenterococcal streptococci. The cost and ease of performance being equal for all of these tests, the use of PYR agar in the presumptive test scheme would seem preferable.

LITERATURE CITED
6. Lancefield, R. L. 1957. Differentiation of group A strep-