Bile-Esculin Test for Presumptive Identification of Enterococci and Streptococci: Effects of Bile Concentration, Inoculation Technique, and Incubation Time

C. Chuard and L. B. Reller

Recognition and differentiation of catalase-negative, alpha-hemolytic and nonhemolytic gram-positive cocci in pairs and chains as enterococci; group D streptococci (mainly Streptococcus bovis); and non-group D viridans group streptococci are clinically important (10). The bile-esculin test is widely used to differentiate enterococci and group D streptococci, which are bile tolerant and can hydrolyze esculin to esculin, from non-group D viridans group streptococci, which grow poorly on bile. First described in 1926 by Meyer and Schonfeld (8), the bile-esculin test was shown by Facklam and Moody (2, 3, 5) to have a sensitivity of 100% and a specificity of 97% for identifying enterococci and group D streptococci. These results were obtained with agar slants containing 4% oxgall (bile salts), inoculated with 1 or 2 drops of a 24-h Todd-Hewitt broth culture of the organism ("next-day" inoculation), and incubated for 48 h. In routine diagnostic bacteriology, such a protocol is impractical, since it requires 3 days from the time colonies are detected on primary plates. Most textbooks and procedure manuals recommend inoculating agar slants directly from a few colonies ("same-day" inoculation) rather than from a 24-h subculture in broth, but data supporting this nonstandardized alternative technique are lacking.

Therefore, we evaluated the sensitivity and specificity of the bile-esculin test with two different methods of same-day inoculation (standardized and nonstandardized) and two different incubation times (24 and 48 h). We also compared esculin slants containing 2 and 4% oxgall in formulations currently available from two major commercial sources in the United States. Catalase-negative, gram-positive cocci in pairs and chains forming alpha-hemolytic or nonhemolytic colonies on 5% sheep blood agar that were positive for PYR (Murex, Dartford, United Kingdom) and grew in tryptic soy broth containing 6.5% NaCl (Becton Dickinson Microbiology Systems [BDMS], Cockeysville, Md.) were identified as enterococci; they were speciated with the API Rapid Strep system (bioMérieux Vitek, Hazelwood, Mo.). Catalase-negative, gram-positive cocci in pairs and chains forming alpha-hemolytic or nonhemolytic colonies that were negative for PYR, did not grow in 6.5% NaCl, were positive for group D antigen by latex agglutination (Murex), and had a suggestive (≥90% probability) biochemical pattern by the API Rapid Strep system were identified as S. bovis. Catalase-negative, gram-positive cocci in pairs and chains forming alpha-hemolytic or nonhemolytic colonies that were negative for PYR and group D antigen (and were insoluble in bile if alpha-hemolytic) were called viridans group streptococci.

A total of 110 enterococcal strains (34 Enterococcus faecalis, 15 Enterococcus faecium, 61 nonhemolytic and nonspecified strains), 30 S. bovis strains (2 alpha-hemolytic and 28 non-hemolytic strains), and 110 non-group D viridans group streptococcal strains (83 alpha-hemolytic and 27 nonhemolytic strains) were tested. The strains were isolated consecutively from blood cultures performed at Duke University Medical Center during a 4-year period, except for 19 S. bovis strains that were obtained from the Mayo Clinic.

Fresh (24-h) bacteria were inoculated on three different esculin agar slants containing either no bile (BDMS), 2% oxgall (equivalent to 20% bile) (BDMS), or 4% oxgall (equivalent to 40% bile) (Remel, Lenexa, Kans.). Except for oxgall, the compositions of the three media were the same. For each medium, the following two inoculation techniques were used: (i) direct, nonstandardized S-shaped inoculation of 1 to 10 colonies and (ii) indirect, standardized inoculation of 10 μl (calibrated loop) of a 0.5 McFarland standard suspension of bacteria in sterile deionized water. The slants were incubated at 35°C in ambient air (2) with loose caps for 48 h. Readings were taken at 24 and 48 h. A reaction was considered positive when one-half or more of the medium was blackened (4).

With one exception, all 110 enterococcal strains gave clear-cut positive reactions after 24 and 48 h of incubation (99% sensitivity). The standardized inoculum (approximately 10^6 CFU) was as sensitive as the heavier, nonstandardized inoculum. Facklam and Moody, using an inoculum of 10^7 to 10^8 CFU on agar slants, reported a sensitivity of 100% at 48 h but found 2 of 76 (5) and 6 of 157 (3) enterococcal strains to be bile-esculin negative (98% sensitivity) after 24 h of incubation. Swan (13), using a nonstandardized inoculum described as heavy as well as agar plates on which any blackening was
streaked on agar slants containing 4% oxgall and read after positive) was maximal (97%) with a standardized inoculum.

We found that the specificity (100% minus the percent false tests for 110 non-group D viridans group streptococcal strains. Regardless of the bile concentration or the method of inoculation (100% sensitivity). Facklam (3) reported a sensitivity of 94 and 100% at 24 h with 121 enterococcal strains. Nonetheless, based on Facklam’s publications, most textbooks and procedure manuals recommend incubation for 48 h before reporting a negative result.

All 30 strains of S. bovis were positive at 24 and 48 h regardless of the bile concentration or the method of inoculation (100% sensitivity). Facklam (3) reported a sensitivity of 94 and 100% at 24 and 48 h, respectively, with 37 group D streptococcal strains.

Table 1 gives the percentages of false-positive bile-esculin tests for 110 non-group D viridans group streptococcal strains. We found that the specificity (100% minus the percent false positive) was maximal (97%) with a standardized inoculum streaked on agar slants containing 4% oxgall and read after 24 h. False positives were obtained with two Streptococcus milleri and one S. lactis subsp. diacetylactis strains. Lack of standardization of the inoculum, decrease in the concentration of oxgall to 2%, and prolongation of the incubation time to 48 h increased the number of false positives to a maximum of 24%. In previous studies with a selective esculin agar containing sodium azide and only 10% bile, positive reactions with non-group D viridans group streptococci were common (6, 11, 12). No data on the use of a medium containing 2% oxgall have been published previously. Our results suggest that this concentration is suboptimal. Using 4% oxgall, Swan (13) found two bile-tolerant viridans group streptococcal strains out of 21 isolates; neither strain, however, hydrolyzed esculin at 24 h. Facklam et al. reported specificities of 99% at 24 h (3) and 81 (4) to 97% (3) at 48 h with 4% oxgall.

A striking difference was found when the subgroups of alpha-hemolytic and nonhemolytic non-group D viridans group streptococci were compared for the number of false positives. For alpha-hemolytic strains, this number was 0% after 24 h and 3% after 48 h, whereas for nonhemolytic strains it was 11% after 24 h and 33% after 48 h, with 4% oxgall and a standardized inoculum (P = 0.017 for 24-h values; two-tailed Fisher’s exact test). Such an observation has not been reported previously.

For specimens other than blood and normally sterile sites, a flowchart based on the bile-esculin test combined with 6.5% NaCl tolerance or presence of PYR is sufficient for reliable identification of enterococci. Bile-esculin-positive organisms from blood and normally sterile sites should be speciated. Speciation of enterococci is useful for epidemiological reasons and because E. faecium and other species tend to be more resistant to antibiotics than E. faecalis (8, 9). A definitive identification of S. bovis is important, since the organism is associated with colonic carcinoma, which should be ruled out in such patients (7). On the other hand, false-positive reports of S. bovis may lead to unnecessary investigations. Speciation of bile-esculin-positive organisms will also allow detection of false-positive non-group D viridans group streptococci. Therapeutic errors can occur with misidentification of streptococci and enterococci (1).

In conclusion, the bile-esculin test works well to rapidly separate enterococci and group D streptococci from non-group D viridans group streptococci at low cost and with good sensitivity (>99%) and specificity (97%), provided it is performed on agar slants containing 40% bile, done with a standardized inoculum (10 μl of a 0.5 McFarland standard bacterial suspension), and read at 24 h.

TABLE 1. False-positive reactions of 110 non-group D viridans group streptococcal strains on esculin slants with 0, 20, and 40% bile

<table>
<thead>
<tr>
<th>Medium</th>
<th>No. (%) of strains with false-positive reactions</th>
<th>24 h</th>
<th>48 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esculin, no bile</td>
<td></td>
<td>42 (38)</td>
<td>56 (51)</td>
<td>42 (38)</td>
<td>56 (51)</td>
</tr>
<tr>
<td>Esculin, 2% oxgall</td>
<td></td>
<td>21 (19)</td>
<td>26 (24)</td>
<td>16 (15)</td>
<td>22 (20)</td>
</tr>
<tr>
<td>Esculin, 4% oxgall</td>
<td></td>
<td>11 (10)</td>
<td>17 (15)</td>
<td>3 (3)</td>
<td>11 (10)</td>
</tr>
</tbody>
</table>

a Direct inoculation of 1 to 10 colonies.

b Indirect inoculation of 10 μl of a 0.5 McFarland standard bacterial suspension.

c Equivalent to 20% bile.

d Equivalent to 40% bile.

e Two S. milleri and one S. lactis subsp. diacetylactis strains. P = 0.049 in comparison with the nonstandardized inoculum (two-tailed Fisher’s exact test).

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REFERENCES


