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

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The bile-esculin test is used to differentiate enterococci and group D streptococci from non-group D viridans group streptococci. The effects on test performance of the concentration of bile salts, inoculum, and duration of incubation were examined with 110 strains of enterococci, 30 strains of *Streptococcus bovis*, and 110 strains of non-group D viridans group streptococci. Optimal sensitivity (>99%) and specificity (97%) of the bile-esculin test can be obtained with a bile concentration of 40%, a standardized inoculum of 10^6 CFU, and incubation for 24 h.

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 esculetin, 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 both 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*, and 61 nonhemolytic and nonspeciated 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

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

Medium	No. (%) of strains with false-positive reactions			
	Nonstandardized inoculum[a]		Standardized inoculum[b]	
	24 h	48 h	24 h	48 h
Esculin, no bile	42 (38)	56 (51)	42 (38)	56 (51)
Esculin, 2% oxgall[c]	21 (19)	26 (24)	16 (15)	22 (20)
Esculin, 4% oxgall[d]	11 (10)	17 (15)	3 (3)[e]	11 (10)

[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).

considered to be a positive result, obtained a sensitivity of 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 *Streptococcus 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). Routine speciation of bile-esculin-negative organisms is not necessary, since enterococci and group D streptococci rarely give false-negative reactions.

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.

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