Improved Procedure for Identification of Group D Enterococci with Two New Media

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With the use of Pfizer selective enterococcus medium as a screening process, tyrosine decarboxylase (TD) medium and D-broth were devised for additional confirmation of the identification of group D enterococci. The semisolid TD medium was used in a method similar to inoculating a motility tube. TD activity was indicated by clarification of the medium near the line of inoculation because the insolubility of tyrosine provided an otherwise milky suspension. D-broth was used to show the tolerance of organisms to 6.5% NaCl at pH 8.2. In addition, enterococcal species (except Streptococcus durans) could also be demonstrated by the acid formation due to the fermentation of mannitol or arabinose. With this improved system, about 97% of enterococci could be accurately identified and confirmed.

There has been a widespread application of bile-esculin medium for the presumptive identification of group D streptococci (3, 6, 7). Nevertheless, other streptococci would also give positive reactions, such as those in group Q, some strains of Streptococcus sanguis, a few strains of S. mitis, and S. mutans (3). In addition, S. bovis and S. sanguis showed antibiotic sensitivity patterns different from other enterococci (8). That is, they were much more sensitive to penicillin and other antibiotics for gram-positive organisms. Therefore, from the clinical viewpoint, non-enterococci should particularly be ruled out from enterococci due to the difference in antibiotic therapy. This would especially concern S. sanguis and S. bovis isolated from blood cultures in subacute endocarditis.

For further confirmation and identification of the enterococcal species, tolerance and sugar fermentation tests were generally recommended (3, 7). But there were some disadvantages in applying these tests. Not only were they difficult to read, but too many tests became time consuming and were therefore not practical for routine laboratory testing.

Tyrosine decarboxylase (TD), on the other hand, has been reported as being a good test for the differentiation of enterococci from other streptococci (1, 2, 4, 5). But the solubility of tyrosine was so low that it has had little value for routine usage. However, the insoluble nature could be used to give a very sensitive test as shown in these studies.

In addition to this TD medium, a D-broth was designed. It was a modification of NaCl broth. The D-broth and the TD medium were designed here for confirmation of the enterococci.

The purpose of this investigation was to evaluate the reliability of these two new media in differentiating group D enterococci from other streptococci.

MATERIALS AND METHODS

Media. For a routine diagnostic bacteriology laboratory, it was convenient to prepare the two media in 200-ml lots. Thus, formula were designed in grams/200 ml.

TD medium. The TD medium was composed of the following: Falkow's decarboxylase basal medium (Difco), 1.8 g; agar (Difco), 0.6 g; and tyrosine (Fisher), 1.0 g. This mixture was evenly suspended in 200 ml of distilled water and autoclaved at a pressure of 15 lb (121°C) for 15 min. The hot medium suspension was shaken well and aseptically distributed in 2-ml amounts into small sterile tubes (10 by 75 mm) with a rubber stopper.

This medium also could be distributed into each tube before autoclaving, if desired. However, it should be boiled previously to dissolve the agar and shaken well to obtain an even suspension of tyrosine. Care should be taken to insure that the rubber stopper is sealed tightly or it will pop out due to the pressure produced by autoclaving. Rubber stopper-sealed tube media can be stored without undergoing
any pH changes much longer than the screw-capped tubes. The medium turned reddish-purple in the refrigerator and then back to purple at room temperature. It could be stored for approximately 1 month, although 2 weeks were preferable.

For use as a semisolid tube medium, the inoculation was done by a single stab to the bottom of the tube. After inoculation, the rubber stoppers could be sealed tightly without overlaying with mineral oil.

**D-broth.** D-broth was composed of the following (in grams): tryptone (Difco), 1.0; Yeast Nitrogen Base (Difco), 0.4; sodium chloride (Fisher), 13.0; mannitol (BBL), 1.0; arabinose (BBL), 1.0; and phenol red (BBL), 0.0005. The mixture was dissolved in 200 ml of distilled water, and the final pH of this medium was adjusted to approximately 8.2 by the use of a 20% NaOH solution. The medium could be distributed into small tubes, either before or after autoclaving, in 2-ml amounts into small tubes (10 by 75 mm) with a rubber stopper. It was autoclaved at a pressure of 15 lb (121 C) for 15 min. The color of this broth should be pinkish-red. Excess autoclaving turns it yellowish-red. Therefore, an alternative way of preparing this medium is by filtration to avoid overheating the sugar. Falcon sterile, disposable filter units (0.22 m, BBL) were used satisfactorily.

**Microorganisms.** One hundred and fourteen strains were isolated from clinical materials. Eleven strains of identified S. sanguis were also included. In addition to these, 252 strains were collected from the Streptococcal Laboratory at the National Center for Disease Control and represented the following species: S. faecalis, 46; S. faecalis var. liquefaciens, 13; S. faecalis var. zymogenes, 20; S. faecium, 12; S. durans, 6; S. bovis, 26; S. sanguis, 45; S. mitis, 74; S. salivarius, 10.

**Inoculation.** All streptococcal strains were first inoculated into 5-ml Todd-Hewitt tubes containing a few drops of rabbit blood and were incubated at 37 C overnight. The following morning they were then transferred to sheep blood plates by streaking to determine hemolysis and purity. Other routine tests included Gram stains and catalysis. Pfizer selective enterococcus medium (PSE) plates, TD tubes, and D-broth were all inoculated from the blood plates. The TD tubes were incubated in a 45 C water bath, whereas the PSE plates and D-broth were both placed in a 37 C incubator. Readings were taken at 24-hr intervals for 3 days.

**Reading the tests.** The TD test was read by holding the TD tubes in front of a light and observing clearing. A clear appearance along the stab of inoculum, without any milky turbidity of the tyrosine, indicated a TD-positive condition. Occasionally, the medium appeared yellowish-purple, with a clear pencil line, and also was interpreted as TD positive. With longer incubation the medium eventually became purple, and then completely clear. This additional length of time was not necessary for reading of the tests, for 24 hr provided sufficient reactions to indicate a TD-positive condition.

However, if the medium turned yellow without clearing, it indicated that the organism grew at 45 C but lacked TD. A negative reaction should be identical to the control, showing purplish turbidity.

D-broth should also be read by holding the tubes in front of light. A positive reaction showed yellowish growth. A positive reaction of the PSE plate was recorded (after 18 hr) as a blackening of the medium.

**RESULTS**

The results of this investigation were compiled to form two tables. Table 1 shows a comparison of the PSE test and the two new media among 263 known strains of group D and non-group D streptococci. As indicated, blackening of the PSE plate appeared from all strains of group D and some strains of "viridans" streptococci, especially those of S. sanguis from clinical blood specimens. However, none of the streptococci, except the enterococci, showed positive reactions in D-broth or produced positive TD. Most of S. bovis, some of S. sanguis, and a few of S. mitis and S. salivarius strains turned the TD medium to yellow; nevertheless, they failed to produce TD and turn the D-broth to a yellowish color. The results, given in Table 1 showed that D-broth and TD tests were 95% accurate for the identification of enterococci.

By using PSE plates as a screening process, 114 PSE-positive isolates from clinical urine specimens were tested with these two new media. As shown in Table 2, the majority of clinical isolates (94%) were positive both with

**Table 1. Reactions of group D and other selected streptococci in three presumptive tests**

| Strains | PSE | D-broth | TD |
|---------|-----|---------|----|
| No.     | %   | No.     | %  | No.     | %   |
| Enterococci* (97) | 97 | 100 | 92 | 95 | 95 | 95 |
| S. bovis* (26) | 26 | 100 | 0 | 0 | 0 | 0 |
| Viridans (140) | 18 | 13 | 0 | 0 | 0 | 0 |

* Group D streptococci.

**Table 2. Reactions of 114 PSE-positive urine isolates on two new presumptive media**

| Reaction patterns* | Test | Strains |
|--------------------|------|---------|
|                    | D-broth | TD medium | No. | Percent |
| 1                  | +      | +        | 107 | 94      |
| 2                  | +      | -        | 3   | 2.6     |
| 3                  | -      | +        | 1   | 0.8     |
| 4                  | -      | -        | 3   | 2.6     |

* Reaction patterns 1, 2, and 3 are with enterococci. Reaction pattern 4 is PSE positive and with non-enterococci.
D-broth and TD. A few strains were D-broth positive only (2.6%) or TD positive only (0.8%). From this study, 97.4% of the isolates could be classified as enterococci, and 2.6% probably belonged to S. bouï or S. sanguis. Thus, with the combined reactions from the two media tube tests, enterococci could be well differentiated from other streptococci.

**DISCUSSION**

**PSE plate.** Isenberge et al. (6) have evaluated some bacteria other than streptococci with PSE medium. In addition, Facklam and Moody (3) have tested their bile-esculin medium with all streptococci from groups A through U. The study here was carried out by first using PSE as an enterococcal enrichment medium for isolation. Then, any suspicious colonies were diagnosed as enterococci through the combined use of TD medium and D-broth.

**TD medium.** That the majority of the enterococci could decarboxylate tyrosine to form the corresponding amine tyramine has been well recognized (4, 5). Furthermore, the differentiation of enterococci from other streptococci, based on the decarboxylase activity of tyrosine, has also been extensively employed (1, 2). Nevertheless, the TD test has not been commonly introduced to the diagnostic laboratory for differentiating enterococci. The main handicap, probably, was the insoluble nature of the tyrosine. If difficulties with the preparation of the medium were overcome, this test would be a very sensitive, convenient test and should be a good test of choice. The majority of enterococci clarify the medium along the stab within 24 hr.

**D-broth.** As is known, major variations with the salt tolerance test depended on inoculum size and degree of enrichment in the broth. A heavy inoculum in an enriched medium with 6.5% NaCl might support growth of many streptococci other than enterococci. However, a light inoculum in a non-enriched medium with 6.5% NaCl would produce a negative result, even for an enterococcus (3).

The readings could be made much easier with D-broth, in the presence of two sugars and with phenol red as an indicator, than with ordinary salt broth. The addition of mannitol and arabinose not only served as an enrichment for this broth, but also offered an extra sugar fermentation test. The majority of enterococci produced satisfactory results in this broth within 24 hr, and prolonged incubation usually was not needed. However, as is known, S. durans would not form acid in arabinose and rarely formed acid in mannitol (1). But this deficiency would be overcome by further testing with the TD medium, for most of that species were TD positive.

The results obtained showed that the application of the PSE plates in conjunction with D-broth and TD medium could be helpful for distinguishing between group D enterococci and other streptococci. The combined use of these two media provide the proper diagnosis. Separate use of only one or the other media results in under-diagnosis.

The conclusion, based on results shown in Table 2 could be summarized as follows: (i) PSE positive, D-broth positive, and tyrosine decarboxylase positive—enterococcus; (ii) PSE positive, D-broth positive, and tyrosine decarboxylase negative—enterococcus; (iii) PSE positive, D-broth negative, and tyrosine decarboxylase positive—enterococcus; and (iv) PSE positive, D-broth negative, and with growth at 45 C—probable S. bouï or S. sanguis.

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