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Evaluation of a New Presumptive Medium for Group D Streptococci

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A new medium designated as D streptococcus-enterococcus broth was formulated and evaluated for the enrichment and isolation of strains of serological group D streptococci. This medium was made by modifying Todd-Hewitt broth. Most-probable-number multiple-tube and membrane filter techniques were employed to estimate the numbers of enterococci in known cultures, wastewater, and other samples. Preliminary most-probable-number counts with this medium were as much as 3 logs higher than those counts obtained from four other media with which it was compared. The methodology for using this medium to estimate the numbers of group D streptococci in water is discussed.

The enterococci of group D, especially *Streptococcus faecalis*, have been used only infrequently or in conjunction with the coliform group as indicators of fecal pollution in the United States. However, these streptococci have been used rather extensively in Great Britain as pollution indexes.

The enterococcus group includes *S. faecalis*, its varieties *S. liquefaciens* and *S. zymogenes*, *S. faecium*, and *S. durans*. These organisms have been used in stream and marine water studies (15, 25) and in shellfish and shellfish-growing water (25, 26) to indicate fecal pollution. Geldreich and Kenner (9) compared the occurrence and persistence of this group with fecal coliforms in domestic sewage, river water, and other types of waste materials. These investigators determined the ratios of fecal coliform to fecal streptococci. The ratio of fecal coliform to fecal streptococci indicating human sewage contamination was determined to be on the order of 4:1. Should a more sensitive medium be developed for the isolation and confirmation of greater numbers of the enterococci, this ratio would be reduced and thus favor the streptococci more significantly.

Presently, the most widely used most-probable-number (MPN) presumptive enterococcus media are Rothe azide dextrose (AD) broth (16), KF broth (14), *S. faecalis* broth of Hajna and Perry (12), and an enterococcus presumptive broth as formulated by Winter and Sandholzer (28).

Mallmann and Seligmann (18) have recommended AD broth as the medium of choice for the quantification of "fecal streptococci" in water, sewage, and food suspected of being contaminated with fecal material. Medrek and Litsky (20) used this same medium to study the incidence of enterococci in soil, reporting a high degree of recovery. Splittstoesser et al. (27) have also reported that AD broth yielded the maximal confirmed numbers of enterococci in frozen foods. *Standard Methods for the Examination of Water and Wastewater* (1) includes AD broth for detecting and estimating numbers of group D streptococci in water by the multiple-tube technique.

The development of a more sensitive medium that allows for the detection and confirmation of the streptococci associated with fecal contamination would be helpful in bacteriological studies in which such information is desired. This report describes the evaluation of such a medium, D streptococcus-enterococcus (DSE) broth, for presumptively detecting these organisms in sewage. The results of an investigation using this medium to estimate the numbers of group D streptococci in samples by both the MPN and the membrane filter (MF) techniques and comparing the sensitivity and selectivity to previously mentioned media are reported.

MATERIALS AND METHODS

Reference cultures. Lyophilized stock cultures from the American Type Culture Collection (ATCC) and other strains of group D streptococci obtained from R. R. Facklam, Center for Disease Control (CDC, Atlanta, Ga.) were used as reference organisms to test the proficiency and sensitivity of all media used in this study. Included were: *S. faecalis*, CDC 277, ATCC 11420, and ATCC 14507; *S. faecalis* var. *liquefaciens*, CDC 275; *S. faecalis* var. *zymogenes*, CDC 499; *S. faecium*, CDC 442; *S. durans*, CDC 661 and ATCC 19432; *S. bovis*, CDC 106; and *S. equinus*, CDC 140 and 1130.

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Media. Azide dextrose broth (AD, Difco), KF broth (Difco), *S. faecalis* broth (BBL), and enterococcus presumptive broth (BBL) were prepared in single-strength concentrations, dispensed in 5-ml amounts in screw-cap tubes (16 by 125 mm), and sterilized by autoclaving (120°C, 15 min, 15 lb/in²).

An experimental medium designated as DSE broth was devised by using Todd-Hewitt broth (THB; Difco) as a base. The medium was prepared as follows: THB, 30 g; NaCl, 5.5 g; dextrose (Difco), 1.0 g; NaN_3, 0.4 g; phenol red, 1.0 ml (1.6 g of indicator per 100 ml of 95% ethanol); distilled water, 1,000 ml. The mixture was dispensed and autoclaved in the same manner as the commercial media. The final pH was 7.6 ± 0.1. Since it has been reported that azide can be lost from media upon heating (13), residual azide was detected qualitatively in sterilized batches of DSE broth by adding 0.2 to 0.3 ml of a 0.1 M solution of $Fe(NO_3)_3$ to at least five tubes from each batch. Azide formed a copper-colored complex.

DSE agar was prepared from the broth by adding 15 g of agar per 1,000 ml of broth. After sterilization and cooling (50°C), 10 ml of a freshly prepared, filter-sterilized 1% solution of 2,3,5-triphenyl-2H-tetrazolium chloride (TTC; Matheson) was mixed with the agar prior to pouring plates. DSE broth that was used in the MF procedure also contained TTC to detect formazan production. Both M-enterococcus (ME) agar (Difco) and KF agar (Difco) were used in MPN and MF tests for the same purpose.

The enterococcus MPN and MF tests and the total and fecal coliform estimates were determined by the procedures and from the statistical tables as described previously (1). In addition, conventional spread plate counts were obtained from pure cultures and from samples by using tryptic soy agar (TSA; Difco).

Tests with known culture. Initial MPN, MF, and spread plate tests were made with an 18-h broth culture of *S. faecalis* CDC 277 to determine the proficiency and rates of recovery of the enterococcal media to be used. Inoculated tubes of DSE broth were incubated at 45°C in a water bath for 24 h. Confirmation of positive tubes was not carried out since a pure culture was used. Plates that contained DSE broth or agar and were used in MF estimations were incubated at either 37 or at 45°C in a water bath for 24 h. All commercial media were treated as specified by the producers.

Sampling and testing. A total of 50 wastewater samples were retrieved from the receiving basin of a water pollution control plant. Ten additional samples were obtained from a local river that received secondarily treated domestic sewage from this same plant. All samples were taken from approximately 12 inches (ca. 30.48 cm) beneath the surface of the water level and transported to the laboratory at ambient temperatures. All tests were initiated within 1 h after collection of the samples.

Each sample was treated in the same manner as the pure culture; i.e., MPN, MF, and plate counts were determined by the procedures described previously (1). Subcultures from positive tubes (yellow color, acid) were streaked onto ME, KF, and DSE agar plates for confirmation. However, only those positive reactions (pink-red colonies) obtained from ME agar were used in calculating confirmed MPN counts to lend uniformity to the comparative methods. Subsequent to streaking on ME agar, three to five colonies were picked and transferred to TSA slants for identification purposes. Enterococcal MF tests, coliform MPN estimates, and plate counts were performed only on the first 10 wastewater samples because the major objective of the study was to test the use of DSE broth as a presumptive medium for the enterococci.

Characterization of isolates. The various biochemical tests used to identify the 300 isolates are described in several investigations (3–8, 11). All fermentation tests, with the exception of glycerol, were incubated aerobically at 37°C for 72 h; glycerol tests were incubated anaerobically at 37°C for 72 h in a glove box at the CDC. Arginine hydrolysis was determined by using Moeller broth (Difco) overlaid with sterile mineral oil. An alkaline reaction (purple coloration) after 18 to 24 h of incubation was considered positive, whereas yellowing (acid) indicated a negative test.

Serological methods. A portion of the isolates (127 strains) was cultured in THB containing 1% glucose as recommended by Medrek and Barnes (19). The extraction by autoclaving method of Rantz and Randall (23) was used to obtain the group-specific carbohydrate. The grouping reaction was determined either by the capillary precipitin test or by the Ouchterlony (22) double-diffusion technique, using group D streptococcal antisera provided by the CDC. Ouchterlony plates were prepared as described by Carpenter et al. (2). Well templates (Penicylinder; 7 by 10 mm; Fisher Scientific Co.) were placed on the agar with their centers 12 mm apart.

RESULTS

Formulation of experimental medium. Various modifications of the experimental medium were tested in preliminary studies to determine which formula would offer the greatest sensitivity and would thus provide for the best detection and isolation of group D streptococci. Media variations found to be inferior were deleted from further consideration.

Glucose was used in concentrations up to 10 g/liter. Results showed that 3 g/liter was sufficient to stimulate or sustain growth of the streptococci and that increasing amounts did not yield any higher numbers of positive tubes.

Phenol red was chosen as the pH indicator because acid production in inoculated tubes was evident in some tests as early as 8 h after incubation. The pH range for positive tubes of DSE broth in the 50 tests was 5.2 to 5.6. By comparison, AD broth (4.2 to 4.5), KF broth (4.2 to 4.8), and *S. faecalis* broth (4.3 to 4.8) had lower and narrower pH ranges. The range for enterococcus presumptive broth (4.8 to 5.2) was slightly higher than the three other commercial media.

Incubation of inoculated tubes of DSE broth and its derivatives was carried out at 37 and 45°C in a water bath for 24 h. Higher MPN values were constantly obtained at the elevated temperature; therefore, 45°C was chosen to be used.

Sodium azide was used as an inhibitor to make the medium more selective since this compound has been shown to inhibit the growth of gram-negative organisms (12, 17, 18). It was noted that when concentrations of less than 0.4 g/liter were used, Gram stains from these tubes revealed organisms other than the desired streptococci.

The phosphate and carbonate concentrations of the broth were varied to give pH values to 9.0. All tests of this nature resulted in lower MPN counts than those obtained by using these salts as originally contained in THB.

Control tests with known cultures. Tests with the group D reference strains gave constant growth and fermentation patterns in DSE broth. All cultures, except the two strains of *S. equinus*, produced acid and formed a precipitous button in the broth after 20 to 24 h of incubation. These two strains did produce acid, but grew as a webby mass throughout the lower half of the broth. It is interesting that none of the 300 isolates demonstrated this characteristic when recultivated in DSE broth. Also, none of the isolates was defined as *S. equinus*.

Table 1 contains data in which MPN, MF, and plate count techniques were used in estimating the numbers of *S. faecalis* CDC 277 in a pure culture. Comparisons showed that excellent agreement was achieved with these three methods for 10 such tests.

MPN results with enterococcal media. Table 2 lists the number of positive tubes and the percentages confirmed when the various presumptive MPN media were used. A greater number of positive DSE tubes was obtained from the higher dilutions of samples than with any of the other media, and, overall, a higher percentage of these tubes was confirmed.

Results from MPN tests on the 50 sewage influent samples, using the various group D streptococcal presumptive media, are given in Table 3. Only ranges and averages are shown for simplification.

In nine tests, MPN counts for DSE broth were $\log^3$ higher than that for any of the other media. Confirmed MPN results from 31 samples showed DSE estimates to be higher by $\log^1$ to $\log^2$. In the remaining 10 tests, other slight differences (less than $\log^1$) were calculated, and

TABLE 1. *Comparison of estimated numbers of S. faecalis in pure cultures by MPN, MF, and spread plate techniques*[a]

Determination	MPN		MF		Spread plate (TSA)
	AD broth	DSE broth	DSE agar	ME agar	
Range	2.4×10^7–4.3×10^{10}	2.4×10^7–4.3×10^{10}	1.8×10^6–6.4×10^8	4.3×10^7–1.0×10^{10}	9.3×10^7–7.1×10^{11}
Avg	(5.1×10^9)	(5.5×10^9)	(1.4×10^8)	(2.9×10^9)	(7.8×10^{10})

[a] All estimates are given as the number of streptococci per milliliters. Counts represent ranges for 10 tests. Parenthetical numbers are averages for the 10 tests.

TABLE 2. *Number of positive MPN tubes obtained per dilution for 50 wastewater samples*

Medium inoculated[a]	No. of positive MPN tubes at a dilution of:					
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
AD broth	147 (98)[b]	117 (97)	56 (98)	27 (91)	5 (60)	0
S. faecalis broth	140 (94)	109 (95)	45 (97)	15 (97)	1 (0)	0
KF broth	148 (99)	126 (98)	96 (98)	52 (98)	34 (100)	12 (92)
Enterococcus presumptive broth	138 (92)	104 (97)	52 (98)	29 (95)	0	0
DSE broth	150 (100)	139 (100)	114 (100)	71 (99)	49 (100)	23 (100)

[a] Three tubes of each broth were inoculated with 1-ml amounts from serial dilutions of each sample. A total of 150 tubes were generated from each dilution from 50 wastewater samples.

[b] Parenthetical numbers represent the percentages of the positive tubes that confirmed on ME agar as containing streptococci.

in one of these tests, KF broth had a higher MPN (9.3×10^5/100 ml) than DSE (1.5×10^5/100 ml). KF broth ranked second to DSE broth in 26 of the tests.

DSE broth and agar were incorporated into the MF test to determine the efficacy of this method in estimating the numbers of streptococci. Such tests were performed on the first 10 wastewater samples only. Table 4 shows that the numbers of colonies obtained with both DSE broth and agar were appreciably lower than with ME agar. Also, all MF counts, regardless of the medium used, were noticably lower than those obtained when DSE broth was used in MPN tests.

Determination of selectivity of DSE broth. Gram stains were made on smears prepared from 150 positive tubes taken from the lowest dilution sets of DSE, and the process was repeated on 150 tubes from the highest dilution groups. Inocula from these same tubes were streaked onto TSA and eosin methylene blue agar plates. In six (4%) of the Gram reactions on cultures from the lowest dilutions, gram-positive bacilli were observed, and organisms belonging to the genus *Bacillus* were isolated in two instances.

Gram-negative rods were not noted in any of the stains, but, in two instances, strains identified as *Escherichia coli* were isolated from the lowest dilution tubes. Only gram-positive streptococci were observed and isolated from the tubes containing the highest dilutions of samples, demonstrating the selectivity of DSE broth for indigenous streptococcal strains.

Total coliform MPN quantities ranged from 1.5×10^5 to 4.3×10^8/100 ml, whereas fecal coliforms were estimated to be from 2.0×10^3 to 9.3×10^7/100 ml. Total plate counts ranged from 4.7×10^5 to 5.6×10^9 organisms/ml. These data showed that high numbers of organisms were present in the background flora and that DSE evidently inhibited them.

Identification of isolates as to species. The first four tests shown in Fig. 1 were used to presumptively identify the group D streptococci. All 300 isolates stained as gram-positive streptococci were negative for catalase activity, blackened bile-esculin medium (BEM), and were tolerant to 40% bile. Although other organisms can give the same reaction pattern, a positive BEM reaction can be used to differentiate group D from non-group D streptococci (7, 8).

The ability of an isolate to initiate growth in broth at 10 and 45°C, or only at 45°C, and in 6.5% NaCl broth was used as a criterion to separate the two enterococcal groups from the non-enterococcal group. On the basis of these tests, a very high percentage of these particular isolates (288/300, 96%) were presumptively categorized as being enterococcal strains. The remaining 12 (4%) were presumed to be non-enterococcal varieties as a result of a positive BEM reaction and failure to grow at 10°C.

Facklam and Moody (7) established that a high degree of correlation exists between the blackening of BEM and a positive precipitin reaction with specific antisera. Gross et al. (11) determined the presence of the group-specific polysaccharide in their strains of *S. bovis*, thus aiding the identification of these isolates.

The precipitin test was helpful in this study. Extracts were obtained only from enterococcal strains because of the limited quantity of grouping sera. Of 127 isolates extracted and

TABLE 3. *Comparison of MPN estimations obtained with enterococcal media*[a]

Medium	MPN range[b]	MPN avg[b,c]
AD broth	4.3×10^3–4.3×10^5	7.6×10^4
S. faecalis broth	9.3×10^2–1.5×10^5	2.3×10^4
KF broth	7.5×10^3–2.4×10^7	1.2×10^5
Enterococcus presumptive broth	4.7×10^3–2.1×10^5	3.6×10^4
DSE broth	1.5×10^5–2.4×10^8	6.1×10^6

[a] MPN estimations are based on confirmation on ME agar.
[b] MPN values are expressed as the number of streptococci per 100 ml.
[c] Average as computed for entire 50 wastewater samples.

TABLE 4. *Comparative ranges and averages of MPN and MF estimations*[a] *on 10 sewage samples*

Determination	No. of organisms/100 ml		
	MPN-DSE[b]	MF-DSE	MF-ME
Range	1.1×10^6–2.4×10^8	1.0×10^4–1.7×10^6	1.5×10^4–5.6×10^6
Avg	(5.3×10^7)[c]	(3.0×10^5)	(1.7×10^6)

[a] All estimates given as organisms per 100 ml.
[b] MPN by using three tubes and DSE broth.
[c] Parenthetical numbers are averages of 10 samples.

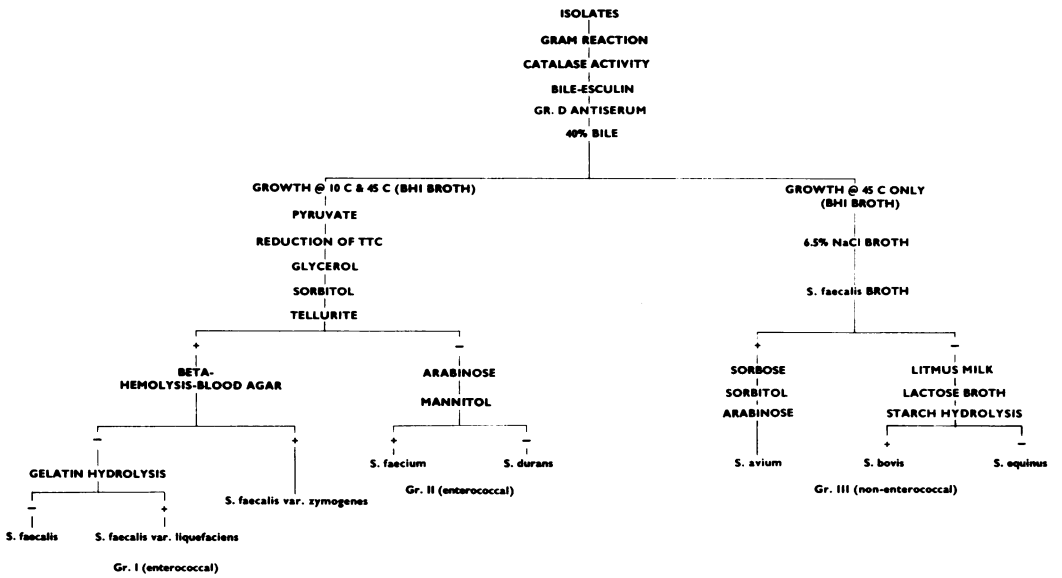

FIG. 1. *Scheme of test protocol used to identify as to species the 300 wastewater isolates obtained from DSE broth.*

tested, 108 gave positive reactions. The 19 strains that yielded negative results were further tested by the Ouchterlony (22) technique. Ten of these tests were positive, whereas nine were still negative; therefore, a high percentage of the isolates tested (118/127, 93%) contained the group D antigen. Figure 2 is a photograph of some of the test results. Isolate 45 was not identified; however, strain 6 was identified as *S. faecalis* var. *liquefaciens* by biochemical tests.

Table 5 contains the results from biochemical tests used to classify the isolates. Of all the tests employed, BEM reactions, precipitin tests, growth at 45 and/or 10°C, tolerance to bile, and growth in 6.5% NaCl broth proved most useful. Melibiose, glycerol, and mannitol fermentations were somewhat beneficial, but the reduction of TTC, the fermentation of arabinose, and the decarboxylation of arginine did not always aid in the identification of certain isolates.

Table 6 shows the species distribution of the isolates. The large majority of the strains were classified; however, 27 organisms were unidentified as a result of their biochemical tests deviating from test patterns of defined group D streptococci.

The non-enterococcus *S. equinus* was not identified among the isolates. *S. faecium* var. *casseliflavus* nov., as described by Mundt and Graham (21), also was not identified. As a matter of interest, none of the 300 strains produced pigment when grown on TSA at room temperature or at 37°C.

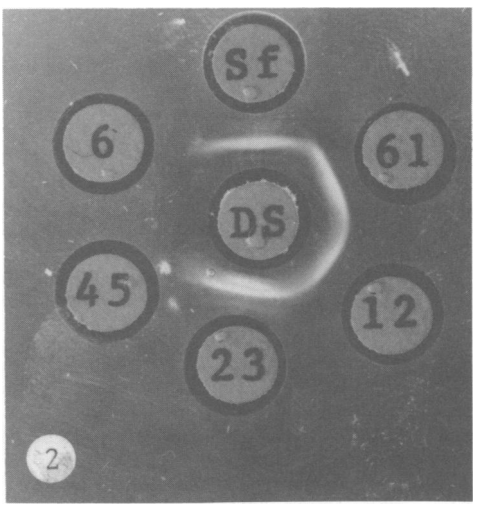

FIG. 2. *Immunodiffusion test results: middle well (DS) = CDC group D-specific antiserum; top well (Sf) = cell wall polysaccharide extract from a known strain of S. faecalis; numbered wells = cell wall extracts from specific streptococcal isolates.*

DISCUSSION

The assumption that THB could be modified and used as a selective enterococcus medium was based on the fact that this medium sup-

TABLE 5. *Biochemical reaction results of sewage isolates*

Test	Enterococci	Non-enterococci
Catalase activity	288/288 (100)[a]	12/12 (100)
BEM reaction	288/288 (100)	12/12 (100)
Group D reaction	118/127 (93)	NTP[b]
Growth on 40% bile	288/288 (100)	12/12 (100)
Growth at 45°C	288/288 (100)	12/12 (100)
Growth at 10°C	288/288 (100)	1/12 (8.3)
Growth in 6.5% NaCl broth	288/288 (100)	2/12 (16.7)
S. faecalis broth	288/288 (100)	6/12 (50)
Reduction of TTC	271/288 (94.1)	3/12 (25)
Hydrolysis of gelatin	16/288 (5.5)	6/12(50)
Hydrolysis of starch	27/288 (9.3)	2/12 (16.7)
Litmus milk		
Acid	231/288 (82)	6/12 (50)
Acid with clot	36/288 (12)	3/12 (25)
Alkaline	12/288 (4)	3/12 (25)
Resistant to tellurite	63/75 (84)	0/12
Resistant to bacitracin[c]	78/80 (96.3)	11/12 (92)
Beta-hemolysis[d]	18/288 (6.2)	1/12 (8.3)
Arginine decarboxylation	61/288 (21)	0/12
Hydrolysis of sodium hippurate	12/288 (4.3)	1/12 (8.3)
Fermentation of:		
Pyruvate	120/288 (41.6)	0/12
Sorbitol	247/288 (85.7)	2/12 (16.7)
Mannitol	280/288 (97)	4/12 (33)
Glycerol	180/288 (62.5)	1/12 (8.3)
Esculin	288/288 (100)	10/12 (83.3)
Lactose	280/288 (97)	3/12 (25)
Raffinose	106/288 (36.8)	4/12 (33)
Arabinose	54/288 (18.8)	0/12
Inulin	179/288 (62)	3/12 (25)
Sorbose	10/288 (3.5)	2/12 (16.7)
Melibiose	51/288 (17.6)	1/12 (8.3)

[a] Numbers in parentheses indicate percentages.
[b] NTP, No tests performed.
[c] This test was not performed on all isolates.
[d] Sheep blood agar plates were used.

ports luxuriant growth of the more fastidious streptococci.

DSE broth gave markedly higher yields than the other media in some MPN tests. These differences could be attributed to one or to several factors. First, THB has a rich protein source and a fermentable sugar, the concentration of the latter being important as shown in initial test results. Second, the relationship of pH to the carbonate-phosphate concentrations affected yields in that, as this buffer system was varied, yields were not as high. Similar effects were obtained by Lachica and Hartman (V. F. Lachica and P. A. Hartman, Bacteriol. Proc., p. 2-3, 1965) as growth of enterococci was enhanced by the presence of bicarbonate at pH 6.0. Possibly, carbonate at pH 7.6 is even more stimulatory to the enterococci.

Finally, the inhibitory effect of azide should be addressed. Azide-dye media are more selective than media containing either ingredient alone (13). In limited studies prior to the present investigation, DSE broth lacking phenol red gave confirmed MPN counts equivalent to media containing both the dye and azide. Hence, it seemed that phenol red was not inhibitory. Contrasting reports are presented in an article by Hartman et al. (13) concerning the loss of azide from media upon heating. Also, Gerencser and Weaver (10) discussed the volatility and toxicity of azide and the effect of pH on the process. These problems, in my opinion, were at least partially circumvented by monitoring for azide in sterilized tubes of DSE broth and by using the medium within 24 to 48 h after its preparation.

Explanations for isolating a greater number of enterococci than non-enterococci may be summarized as follows: reference strains of *S. bovis* and *S. equinus* grew well in DSE broth after passage through enrichment broth, whereas like species from environmental samples might have been injured or stressed and ultimately lost; ME agar may be too selective for certain, if not most, non-enterococci, since it has been shown to be practically 100% selective for the enterococci, and more enterococci are expected to be indigenous to domestic sewage.

TABLE 6. *Identification of sewage isolates as to species from resultant biochemical and serological tests*

Organism	PF[a]	DPF[b]
S. faecalis	63/124 (51)[c]	61/124 (49)
S. faecalis var. *liquefaciens*	50/57 (87)	7/57 (13)
S. faecalis var. *zymogenes*	11/16 (69)	5/16 (31)
S. faecium	45/56 (80)	11/56 (20)
S. durans	11/15 (73)	4/15 (27)
S. avium	2/4 (50)	2/4 (50)
S. bovis	0/2 (0)	2/2 (100)

[a] Biochemical test results matched those of the control organism. PF, Perfect fit.
[b] DPF, Deviation from perfect fit. Biochemical test pattern of the isolate varied from that of the control organism by at least one, but by as many as four tests.
[c] Numbers in parentheses indicate percentages.

Regardless of the medium used, MF recovery rates were appreciably lower than those obtained from DSE broth in MPN tests. Hence, the necessity for preenrichment with a selective broth preceding the use of a more stringent confirmatory medium is emphasized.

The results from this study demonstrated that DSE broth can be useful in detecting and estimating the numbers of group D streptococci in wastewater or possibly from various other specimens in which there is a mixed flora. Further studies with many environmental strains from a variety of water sources are necessary to determine the true sensitivity and usefulness of this medium.

LITERATURE CITED

1. **American Public Health Association.** 1976. Standard methods for the examination of water and wastewater, 14th ed. American Public Health Association, New York.
2. **Carpenter, D. H., J. S. Garvey, N. E. Cremer, and D. S. Sussdorf.** 1964. Antigen-antibody reactions, p. 143-149. *In* L. Stevens (ed.), Methods in immunology. W. A. Benjamin, Inc., New York.
3. **Deibel, R. H.** 1964. The group D streptococci. Bacteriol. Rev. 28:330-366.
4. **Deibel, R. H., and C. F. Niven, Jr.** 1964. Pyruvate fermentation by *Streptococcus faecalis*. J. Bacteriol. 88:4-10.
5. **Facklam, R. R.** 1972. Recognition of group D streptococcal species of human origin by biochemical and physiological tests. Appl. Microbiol. 23:1131-1139.
6. **Facklam, R. R.** 1973. Comparison of several laboratory media for presumptive identification of enterococcal and group D streptococci. Appl. Microbiol. 26:138-145.
7. **Facklam, R. R., and M. D. Moody.** 1970. Presumptive identification of group D streptococci: the bile-esculin test. Appl. Microbiol. 20:245-250.
8. **Facklam, R. R., J. F. Padula, L. G. Thacker, E. C. Wortham, and B. J. Sconyers.** 1974. Presumptive identification of group A, B, and D streptococci. Appl. Microbiol. 27:107-113.
9. **Geldreich, E. E., and B. A. Kenner.** 1969. Concepts of fecal streptococci in stream polution. J. Water Pollut. Control Fed. 41(Suppl.): R-336-352.
10. **Gerencser, V. F., and R. H. Weaver.** 1959. A new technique for the use of sodium azide (hydrazoic acid) as an inhibitive agent. Appl. Microbiol. 7:113-115.
11. **Gross, J. C., M. P. Houghton, and L. B. Senterfit.** 1975. Presumptive speciation of *Streptococcus bovis* and other group D streptococci from human sources by using arginine and pyruvate tests. J. Clin. Microbiol. 1:54-60.
12. **Hajna, A. A., and C. A. Perry.** 1943. Comparative study of presumptive and confirmative media for bacteria of the coliform group and fecal streptococci. Am. J. Public Health 33:550-556.
13. **Hartman, P. A., G. W. Reinbold, and D. S. Saraswat.** 1966. Media and methods for isolation and enumeration of the enterococci. Adv. Appl. Microbiol. 8:253-289.
14. **Kenner, B. A., H. F. Clark, and P. W. Kabler.** 1960. Fecal streptococci. I. Cultivation and enumeration of streptococci in surface waters. Appl. Microbiol. 9:15-20.
15. **Levin, M. A., J. R. Fischer, and V. J. Cabelli.** 1975. Membrane filter technique for enumeration of enterococci in marine waters. Appl. Microbiol. 30:66-71.
16. **Litsky, W., W. L. Mallmann, and C. W. Fifield.** 1953. A new medium for the detection of enterococci in water. Am. J. Public Health 43:873-879.
17. **Mallmann, W. L., W. E. Botwright, and E. S. Churchill.** 1941. The selective bacteriostatic effect of slow oxidizing agents. J. Infect. Dis. 69:215-217.
18. **Mallmann, W. L., and E. B. Seligmann, Jr.** 1950. A comparative study of media for the detection of streptococci in water and sewage. Am. J. Public Health 40:286-289.
19. **Medrek, T. F., and E. M. Barnes.** 1962. The influence of the growth medium on the demonstration of a group D antigen in faecal streptococci. J. Gen. Microbiol. 28:701-709.
20. **Medrek, T. F., and W. Litsky.** 1960. Comparative incidence of coliform bacteria and enterococci in undisturbed soil. Appl. Microbiol. 8:60-63.
21. **Mundt, J. O., and W. F. Graham.** 1968. *Streptococcus faecium* var. *casseliflavus*, nov. var. J. Bacteriol. 95:2005-2009.
22. **Ouchterlony, O.** 1948. *In vitro* method for testing the toxin-producing capacity of diphtheria bacteria. Acta Pathol. Microbiol. Scand. 25:186-201.
23. **Rantz, L. A., and E. Randall.** 1955. Use of autoclaved extracts of hemolytic streptococci for serological grouping. Stanford Med. Bull. 13:290-291.
24. **Slanetz, L. W., and C. H. Bartley.** 1957. Numbers of enterococci in water, sewage, and feces determined by the membrane filter technique with an improved medium. J. Bacteriol. 74:591-595.
25. **Slanetz, L. W., and C. H. Bartley.** 1965. Survival of fecal streptococci in sea water. Health Lab. Sci. 2:142-148.
26. **Slanetz, L. W., C. H. Bartley, and K. W. Stanley.** 1968. Coliforms, fecal streptococci and salmonella in sea water and shellfish. Health Lab. Sci. 5:66-78.
27. **Splittstoesser, D. F., R. Wright, and G. J. Hucker.** 1961. Studies on media enumerating enterococci in frozen vegetables. Appl. Microbiol. 9:303-308.
28. **Winter, C. E., and L. A. Sandholzer.** 1946. Recommended procedure for detecting the presence of enterococci. TL2, U.S. Department of the Interior, Fish and Wildlife Service, Washington, D.C.