Enterococci may be more useful as indicators of water pollution than the coliform organisms. The study reported upon here is the last of a long chain of efforts to perfect suitable test media for these indicators and proposes both a presumptive and a confirmatory medium.

A New Medium for the Detection of Enterococci in Water*

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THERE is a need for a test organism other than the coliform group for the detection of fecal contamination in foods, water, soil, and other materials. The coliforms have been employed for measuring pollution because these bacteria are supposedly indicative of sewage contamination and can be easily isolated and confirmed by a relatively simple bacteriological technic.

There are many instances in the examination of samples of materials suspected of sewage contamination where the coliform organisms, because of their ubiquity in nature and their ability to multiply outside the animal body, fail to measure sanitary quality. The coliform organisms do not always represent pollution because these organisms are found occasionally in uncontaminated soil and water and may be of nonfecal origin. They may persist in soil and water for long periods of time and may thus not indicate recent sewage pollution. Fecal strains of coliform bacteria cannot be distinguished from nonfecal strains.

Streptococci are used in a limited manner as indicators of pollution on the same basis as coliform organisms. They are present in feces, sewage, and contaminated water; they are not found in potable waters, virgin soils, and sites out of contact with animal and human life, and they do not multiply outside the animal body except in a rich nutrient menstruum.

With respect to the number of streptococci in feces and sewage, a review of literature reveals that while at times they may be almost as numerous as the coliform organisms, at other times they may be considerably less or absent. These variations are due in part, at least, to failure of the methods or media used for the measurements of their frequency.

Mallmann [1] reported that the streptococci were constant indicators of pollution and the number found in swimming pools was parallel to the amount of pollution as indicated by the number of bathers. In a later paper, Mallmann and Sypien [2] compared the coliform and streptococci indexes of samples taken five feet from the shore

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on a bathing beach. It was found that while the coliform indexes and total plate counts did not always respond to changes in the bathing load, the streptococci indexes did. The streptococci were not found at points free from the bathing pollution, while the coliform bacilli were. It was also reported that the streptococci disappeared overnight while the coliform index and the total count sometimes increased, although they were generally lower.

Ritter and Treece [3] isolated 79 strains of streptococci from swimming pools. Fifty-two or 65.8 per cent were classified as *Streptococcus faecalis*; these were confirmed by the Lancefield technic and were further classified as type D.

Winter and Sandholzer [4] confirmed the work of Mallmann and Sypien in that they reported that coliform organisms persisted for a great distance from the source of pollution in water but that streptococci did not. They reported that while streptococci were present in all samples of human and animal feces tested, these organisms were never found in virgin soils or in soils from wooded areas.

Mallmann and Litsky [5] using a dextrose azide broth as an enrichment medium could not isolate enterococci from soils which were not treated with sewage or animal manure. They also stated that other than the coliform organisms, the enterococci were the only organisms found in feces that could be used as indicators of fecal pollution. While the coliform organisms were found to persist in sewage treated soil, the enterococci were found to die out rapidly, but not as rapidly as virulent typhoid bacilli. It was also noted that the longevity of these three organisms in sewage treated soil was prolonged with an increase of the organic content of the soil.

In 1906 Prescott and Baker [6] reported that when *Escherichia coli* and streptococci were grown in mixed cultures, *E. coli* reached a maximum growth before the streptococci but were gradually displaced by the latter 20–60 hours after the start of the experiment. At this time on the streptococci predominated, and, on some occasions, the *E. coli* disappeared completely.

Since similar successions of growth occur in lactose broth, Mallmann and Gelpi [7] suggested the use of standard lactose enrichment broth for the detection of streptococci. After the usual coliform confirmation tests were made, the tubes were reincubated for 48 hours, centrifuged, and the sediment examined for streptococci by microscopical examination. Another method suggested by these authors was to allow the tubes to remain at normal temperature for 1–3 days after the initial incubation to permit sedimentation. It was reported that a heavy sediment in the bottom of the tube, similar in appearance to the deposition in a positive macroscopic agglutination test, was an indication of the presence of streptococci but it must be confirmed by microscopic examination.

In 1918 Weissenbach [8] was one of the first to describe a selective medium for enterococci. Sterile filtered ox bile was used for the inhibitory agent. Baggar [9] also used sterile ox bile with one per cent peptone to grow fecal streptococci. For confirmation he suggested a heat resistant test as recommended by Huston.[10]

Fleming [11] in 1932 reported that enterococci will grow in a concentration of 1–15,000 potassium tellurite which is inhibitory to coliform bacteria as well as other Gram-negative bacteria. Harold [12] in 1936 prepared a solid tellurite agar. Enterococci appeared as bluish-black colonies about a millimeter in diameter with a peripheral opalescence. In 1937 Harold [13] compared this medium to those used at that time and found that from a series of over 250 positive-MacConkey broth tubes, 13.9

per cent contained fecal streptococci by Huston's heating technic, 30.6 per cent by the direct tellurite method, and 44 per cent by the enrichment tellurite method.

Hartman [14] in 1937 was the first to use sodium azide to suppress the growth of Gram-negative bacteria while permitting the streptococci to grow. Since this discovery, many investigators have used this chemical in media for the isolation of fecal streptococci.

Mallmann [15] reported a medium containing sodium azide which was found useful in estimating the number of streptococci in feces as it was found to support the growth of these bacteria while inhibiting the coliform group. Hajna and Perry [16] published another selective streptococci medium which was almost a duplicate of the medium suggested by Mallmann,[15] but the former workers used an incubation temperature of 45° C. Growth and the production of acid in this medium were stated to be almost complete evidence of the presence of *S. faecalis*.

Winter and Sandholzer [17] in 1946 published a procedure for detecting the presence of enterococci. This method consists of a sodium azide presumptive broth and a penicillin-methylene blue sodium chloride broth slant confirmation medium. This method, however, must be confirmed by a microscopical examination and a catalase test.

Chapman [18] in 1944 published formulas of two media for the isolation of streptococci in mixed cultures. Tellurite streptococci medium contained crystal violet, trypan blue and sodium tellurite as active agents. On this medium, *S. salivarius* produces pale blue opaque colonies from 2–5 mm. in diameter, *S. mitis* produces blue colonies about 0.2 mm. in diameter, while the enterococci give a dark brown or sometimes black, slightly raised colony from 0.5 to 1.5 mm. in diameter. Ninety-seven per cent of the transplants from feces were streptococci; the remainder were staphylococci. The azide violet blue medium contained S.T. 37, sodium azide, and crystal violet as inhibitory agents. In this medium *S. mitis* grows in colonies producing orange halos; enterococci produce large blue colonies with halos in some; while *S. salivarius* produces no halo. It was reported that with the increase of sodium azide in this medium, more coliform bacteria were inhibited but the size and number of streptococci colonies were also reduced.

In 1946 Chapman [19] reported a medium for the isolation of enterococci. This was called mitis-salivarius agar—a modification of a former medium. Enterococci produced a dark or black, slightly raised colony about a millimeter in diameter as contrasted to a blue (gum drop) colony of *S. salivarius* or the minute colony of *S. mitis*.

Mallmann and Seligmann,[20] in a comparative study of media for the detection of streptococci in water and sewage, studied the following media: lactose broth, azide broth (Mallmann), S.F. broth (Hajna and Perry), and azide dextrose broth (Rothe). The results of this study were as follows:

Average streptococci indexes of river water:

Medium	Number of Streptococcus per 100 ml.
Lactose broth	930
Azide broth (Mallmann)	3,700
S.F. broth (incubated at 45° C.)	600
Azide dextrose broth (Rothe)	9,200

The authors stated that the positive azide glucose tubes should be checked microscopically because some Gram-positive rods may show turbidity equally as good as the streptococci. They suggest azide dextrose broth as a new means of testing for and measuring streptococci in water, sewage, and shellfish, as well as other materials suspected of sewage pollution.

As a result of the studies made by Mallmann and Seligmann,[20] it was de-

cided that azide dextrose broth (Rothe) was a satisfactory enrichment medium to obtain a presumptive test for streptococci. In the studies presented, an attempt has been made to develop a confirmatory medium so that an enterococci index could be obtained in a manner similar to that used for the detection of coliform according to the present standard procedures for water analysis.

In order to formulate a confirmatory medium for enterococci a base medium first had to be perfected that would not only support the growth of a minimal seeding of these organisms but would also give a short lag phase in the development of the resting cells.

The procedure for determining the growth rate of *S. faecalis* in various test media was that used by Darby and Mallmann [21] and is as follows: A 24-hour culture, which was transferred each day for four days in brain-heart infusion broth, was diluted so that between 20 and 100 organisms per ml. were present. A seeding of this density was planted into flasks that contained 100 ml. of the test medium. Initial plate counts were made immediately after the original seeding and every three hours thereafter, up to and including the ninth hour of incubation. The flasks were incubated at 37° C. and shaken for two minutes prior to each sampling. A dextrose agar with the same base ingredients as that used in dextrose azide agar was used. All plates were incubated at 37° C. for 24 hours.

Six formulations of media were tried with five separate trials. The best results were obtained with a medium containing the following ingredients:

	Per cent Composition
Tryptose (Difco)	2.0
Dextrose	1.50
NaCl	0.50
K_2HPO_4	0.27
KH_2PO_4	0.27
Sodium azide	0.02

Media containing 1.5 and 3.0 per cent salt were found to be slightly inhibitory.

In an attempt to increase the inhibitory action of the medium for Gram-negative bacteria, as well as to determine the limiting concentration for the enterococci, media were prepared with concentrations of sodium azide ranging from 0. to 0.05 per cent.

These media were tested by growth curve rates as previously described. The results indicated that the growth peak for the control (no sodium azide) was 18 hours; whereas, the growth peaks for media containing 0.02–0.03 per cent sodium azide were 42 hours and for media containing 0.4–0.5 per cent were 48 hours or more. The medium containing 0.02 per cent sodium azide inhibited the coliform organisms.

The medium containing 0.04 per cent sodium azide was selected on the basis that enterococci tolerated this concentration with good growth in 48 hours. Inasmuch as the medium was to be used as a confirmatory test, the organisms transferred to this medium would be in their logarithmic growth phase and a large number of cells would be used. For this reason the medium could actually be slightly toxic to resting cells of enterococci.

When this medium was used as a confirmatory test for enterococci on sewage contaminated river samples, the Gram-negative bacteria were inhibited but some spore-formers, such as *Bacillus subtilis* grew. For this reason the addition of another inhibitory agent was necessary to remove Gram-positive bacteria, other than enterococci.

A bacteriostatic dye inhibitory to Gram-positive bacteria, ethyl violet, has been reported by Litsky, Mallmann, and Fifield.[22] They report that this dye is equal to crystal violet and brilliant green in bacteriostatic action against Gram-positive spore-formers but is far less toxic toward Gram-negatives, such as *E. coli* and the Salmonella group.

This dye along with crystal violet and brilliant green was tested for inhibitory action against *S. faecalis*, *B. subtilis*, and *Micrococcus pyogenes* var. *aureus* by checking for complete inhibition or uninhibited growth both in liquid and solid media in 48 hours at 37° C. At a dilution of 1–800,000 for crystal violet and 1–1,200,000 for ethyl violet, *B. subtilis* and *M. pyogenes* var. *aureus* were completely inhibited and *S. faecalis* grew without apparent inhibition.

When confirmatory media were prepared using the above mentioned dyes at the designated dilution, and tested on contaminated river water, the ethyl violet azide broth gave the best results. For this reason, the ethyl violet formula was adopted for further work. The formula was as follows:

	Grams per Liter
Tryptose	20.0
Dextrose	15.0
NaCl	5.0
K_2HPO_4	2.7
KH_2PO_4	2.7
Sodium azide	0.4
Ethyl violet	0.00012

The medium is sterilized at 121° C. for 15 minutes.

Ethyl violet is an uncertified dye so the dye content varies from one batch to another. In the original studies National Aniline and Dye Company Lot 3330 with a dye content of 64.7 per cent was used. Later media have been prepared with Lot 12552 with a dye content of 57.5 per cent.

The specificity of the medium for enterococci was determined by seeding the medium with a number of cocci and incubating for 48 hours and examining the tubes for turbidity.

The cultures selected were as follows:

Number of Isolates	Name of Organism	Growth
8	*S. faecalis*	+
1	*S. durans*	+
1	*S. liquefaciens*	+
1	*S. zymogenes*	+
3	*S. salivarius*	—
4	*S. mitis*	—
1	*S. bovis*	—
1	*S. equisimilis*	—
1	*S. lactis*	—
1	*S. thermophilis*	—
1	*S. agalactiae*	—
1	*S. uberis*	—
1	*S. canis*	—
1	*S. equi*	—
24	*M. pyogenes* var. *aureus*	—
1	*M. pyogenes* var. *albus*	—
1	*M. pyogenes* var. *agilis*	—
1	*Sarcina lutea*	—
1	*Sarcina citrea*	—
1	*Serratia marcescens*	—
Other organisms tested		
1	*Proteus vulgaris*	—
1	*E. coli*	—
1	*E. coli* var. *communior*	—
1	*Salmonella typhimurium*	—
1	*Salmonella typhosa*	—
1	*Shigella alkalescens*	—
1	*B. subtilis*	—
1	*B. cereus*	—
1	*Alkaligenes faecalis*	—
1	*Chromobacter violaceum*	—

The results are particularly interesting in that the medium is specific for enterococci. It is also interesting to note that all Gram-negative and positive bacteria tested other than the enterococci are inhibited. It will be observed that all four species of enterococci grew equally well.

In order to test the effectiveness of ethyl violet azide broth as a confirmatory medium for the detection of enterococci, samples of water were collected from a river at points of little sewage contamination and at points below the outfall of a sewage effluent from a complete treatment sewage plant. Appropriate dilutions of the river water were added to dextrose azide broth and incubated at 37° C. for 48 hours. Three loopfuls of the positive dextrose azide broth tubes were seeded into ethyl violet azide broth which was incubated at 37° C. for 48 hours. Microscopic examinations were made from all positive dextrose azide and ethyl violet azide broth tubes after 48 hours incubation. In one series, 164 samples were tested and in another series six months later 50 samples were checked.

The need of confirming the positive dextrose azide broth presumptive tests was clearly indicated by the fact that in 50 samples examined, the average MPN index was 1,350 and the average presumptive MPN index was 5,370. The "false-positives" obtained in the presumptive test is due mostly to the growth of Gram-positive bacilli and to some extent to Gram-positive cocci other than enterococci. Mallmann and Seligmann [20] observed that the dextrose azide broth was selective for streptococci only in the absence of Gram-positive rods in the test material. River water generally carries a relatively high population of bacilli.

Since some streptococci were found microscopically in the 48-hour dextrose azide broth tubes, but were not confirmed in the ethyl violet azide broth, tests were made to determine whether these organisms were of fecal or non-fecal origin. Tubes which were not confirmed were diluted by loop dilution and plated on brain-heart infusion agar. After incubation at 37° C. for 24 hours, discrete colonies were fished and planted into tryptose phosphate broth. After purity was established these cultures were tested for growth at 45° C. and in 6.5 per cent sodium chloride broth. Of 82 cultures checked, seven were classified as fecal and five of them now grew in ethyl violet azide broth. These "false-negatives" in ethyl violet azide broth may have been due to insufficient inoculum or to atypical strains.

Out of 277 positive ethyl violet azide tubes examined microscopically, two showed Gram-positive rods alone and two showed Gram-positive rods and chains of streptococci. The Gram-positive rods do not appear in the dextrose azide tubes in the low dilutions where enterococci are present. They appear in the high dilutions only when the incidence of Gram-positive bacilli is greater than the enterococci. In the river water with a low sewage contamination, Gram-positive rods frequenty appeared in the 0.01 ml. dilutions. It is interesting to note that in only two cases growth occurred where dextrose azide tubes containing Gram-positive rods were transferred to ethyl violet azide broth.

TABLE 1

The MPN of Enterococci Which Were Detected and Confirmed by Various Methods

Sample Number	*Perry's S. F. Method*	*Winter and Sandholzer's Method*	*Dextrose Azide and E. V. A. Broth Method*
1	91	0	920
2	91	0	430
3	36	36	430
4	36	0	740
5	240	0	920
6	91	0	150
7	0	0	0
8	0	0	0
9	0	0	0
10	0	0	0
11	0	0	73
12	0	0	73
13	0	0	480
14	0	0	240
15	0	0	920
16	0	36	430
17	0	0	9,200
18	0	0	2,500
19	0	73	140
20	0	91	920
21	0	240	240
22	91	150	2,500
23	240	1,470	4,300
24	7.3	36	91
25	0	90	1,500
26	40	150	700
27	91	210	4,500
28	0	70	4,500
29	0	450	2,000
30	40	450	25,000
31	240	1,500	20,000
32	91	450	25,000
33	0	3,000	110,000
34	0	40	2,500
35	0	0	15,250
36	91	40	15,000
37	0	40	35,000
38	40	40	20,000

Although as indicated, in a few instances enterococci growing in dextrose azide broth failed to grow when transferred to ethyl violet azide medium, and in a few instances, growth in the latter medium was due to Gram-positive rods. The procedure for determining enterococci quantitatively by a presumptive

test in dextrose azide broth followed by a confirmatory test in ethyl violet azide medium appears to be a satisfactory method.

To compare the dextrose azide-ethyl violet azide broth method for the detection and confirmation of enterococci with other methods that have been recommended, samples of river water and sewage were planted into these media and confirmation procedures carried out as previously described.

The dextrose azide-ethyl violet azide broth procedure was compared with the Winter and Sandholzer method and the Hajna and Perry procedure using samples of river water and sewage. Suitable dilutions were planted in parallel. The results are presented in Table 1. In every case, with the exception of sample 21, the dextrose azide-ethyl violet azide procedure detected and confirmed 100–1,000 as many enterococci as did the other two methods. Samples 6 to 12 were taken from a relatively unpolluted stream. Indexes of 73 were obtained in two samples, Numbers 11 and 12, with dextrose azide-ethyl violet azide method, but the other two methods failed to show any enterococci in any of the six samples.

SUMMARY

A selective medium containing ethyl violet and sodium azide is presented that is specific for the growth of enterococci from pure cultures or from glucose azide broth showing growth from sewage contaminated water.

A new test for enterococci is proposed wherein glucose azide broth is used as a presumptive medium and ethyl violet azide broth as a confirmatory medium.

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