The membrane filter technique described holds promise for the more extensive use of enterococci as indicators of the sanitary quality of water and other materials.

Use of the Membrane Filter Technique To Enumerate Enterococci in Water

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ALTHOUGH tests for coliform organisms are still generally used to determine the sanitary quality of water, there continues to be much interest in the use of enterococci as indicators of fecal pollution. Considerable evidence is available to show that the presence of enterococci in water or in other materials may more accurately indicate fecal contamination than the presence of coliforms since it is difficult to establish the fecal origin of the latter organisms. A number of investigators have shown that the enterococci are present in feces, sewage, and polluted water and that they are not found in water, soil, or other materials free from human or animal contacts or contamination.

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As early as 1900, Houston (*1*) demonstrated that streptococci are present in polluted waters and appeared to be absent in nonpolluted samples.

When Mallmann and Sypien (*2*) in 1934 compared the coliform and streptococcus indexes of samples of water taken 5 feet from the shore of a bathing beach, they found that while the coliform indexes and total plate counts did not always respond to changes in bathing loads, the streptococcus indexes did. The streptococci were not found at points free from bathing pollution although the coliform organisms were present in such areas.

Winter and Sandholzer (*3*) also reported that coliform organisms persisted for a great distance from the source of pollution in water, but that the streptococci did not. They found that although 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.

Ostrolenk and Hunter (*4*) demonstrated that in 37 percent of 51 fecal specimens which they examined, enterococci occurred in equal or greater numbers than did *Escherichia coli*. In the remaining 63 percent of the specimens, *E. coli* exceeded enterococci numerically by from 1 to 5 decimal dilutions. These investigators suggested that the lower number of enterococci in

human and animal feces does not necessarily minimize the potential sanitary significance of fecal streptococci.

Mallmann and Litsky (*5*), using a dextrose azide broth, were unable to isolate enterococci from soils which were not treated with sewage or animal manure. Although the coliforms were found to persist in sewage-treated soil, the enterococci were found to die out rapidly but not as rapidly as virulent typhoid bacilli.

The lack of suitable methods and media for the detection and estimation of the numbers of enterococci in water or other substances has been one of the chief problems in the use of these bacteria as indicators of fecal pollution. This situation may also account for variations in results reported in the estimation of numbers of these organisms in fecal specimens or in water.

In recent years, much progress has been made in the improvement of techniques for the cultivation of these organisms from various materials. Important contributions have been made by Mallmann (*6*), Winter and Sandholzer (*3*), and others (*7–11*). Because many of these papers have been reviewed briefly by Litsky, Mallmann and Fifield (*11*), they will not be discussed here.

Mallmann and Seligmann (*10*) compared standard lactose broth, sodium azide broth (Mallmann), Hajna and Perry S.F. (*Streptococcus faecalis*) broth (Difco), and Rothe azide dextrose broth (Difco) as media for the detection of streptococci in water and sewage. Mallmann and Seligmann noted that the Rothe azide dextrose broth gave the best results for the quantitative determination of streptococci. However, the tubes had to be checked microscopically since gram-positive bacilli might be responsible for turbidity in the cultures. They suggested that azide dextrose broth be used as a new means for testing and measuring streptococci in water, sewage, and shellfish, or in other materials suspected of sewage pollution.

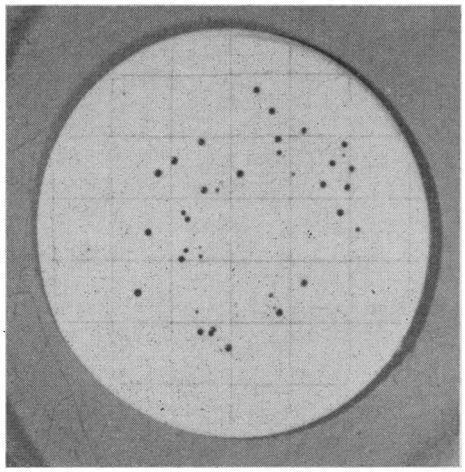
Litsky, Mallmann, and Fifield (*11*) attempted to develop a confirmatory medium so that an enterococcus index could be obtained in a manner similar to that used for coliforms by the present standard methods procedures for water analysis. These investigators were able to prepare a medium containing ethyl violet and sodium azide, a medium which they found highly selective and specific for the growth of enterococci. They proposed a new test for enterococci which would use the dextrose azide broth as a presumptive medium and the ethyl violet azide broth as a confirmatory medium. The dextrose azide and ethyl violet broth procedure detected and confirmed 100 to 1,000 times as many enterococci as did the Hajna-Perry S.F. method and the Winter-Sandholzer procedures.

In the present study, our interest was in determining whether, by using the membrane filter technique, methods and media could be developed for the detection and enumeration of enterococci in water. Efficient filter techniques for detecting coliforms in water had been reported by Clark and associates (*12*), Goetz and Tsuneishi (*13*), Kabler (*14*), and others, and there is also a report now in press (*15*). Thus, it was thought that similar procedures might prove valuable for determination of enterococci.

Materials and Methods

Two types of membrane filter—Millipore (*A*) and Bac-T-Flex (*B*)—and the apparatus supplied for both types were used in these studies. The filters were sterilized by placing them bebetween S&S absorbent pads (*B*), wrapping them in desired numbers in kraft or some other type of wrapping paper, and autoclaving at 121° C., at 15 pounds pressure, for 10 minutes. Then the steam pressure was reduced rapidly to prevent condensation of water on the membranes. When ready for use, the filters and pads were handled by placing them in sterile petri dishes. The filter apparatus was sterilized by autoclaving at 121° C. for 10 minutes also.

For the selective cultivation of enterococci, various media and inhibitory substances were tested for use with the membrane filters. These included: the Hajna-Perry S.F. broth; the Rothe azide dextrose broth; the Winter-Sandholzer sodium azide presumptive broth; the Chapman mitis-salivarius medium; the confirmatory broth of Litsky, Mallmann, and Fifield; and modifications of all these media. Each medium was also tested after the concentration of the nutrient materials was doubled.



Colonies of enterococci on a Bac-T-Flex filter. This picture has not been magnified. One hundred milliliters of polluted river water was filtered through this membrane before it was incubated on pads with the selective enterococcus medium.

To color the colonies of enterococci, 0.01 percent 2,3,5 triphenyl tetrazolium chloride (TTC) was added to the media. A stock solution of 1 percent of the TTC was prepared and sterilized at 121° C. for 15 minutes. Then just before use it was added aseptically in the required amounts to the broth media.

The filtration and cultivation procedures used during these studies were similar to those outlined for determination of coliform bacteria in water (*12–15*). The various culture media tested were added in 2.2-milliliter amounts to the absorbent pads. The desired amount of water sample or culture material was filtered through the membrane filters using a vacuum produced by a filter pump on water pressure. The filters were then transferred directly to pads containing the test media. These cultures were placed on a shelf about 1 inch above the water level in a covered water bath and incubated at 35° to 37° C. for 48 hours. After incubation was completed, colony counts were made using a stereoscopic microscope magnifying 10 times.

Cultivation of Enterococci on Filters

To determine the efficiency of various media for the cultivation of enterococci on the membrane filters, both dilute suspensions of *Streptococcus faecalis* and samples of polluted water were employed. Of the different media listed in the preceding section on "Materials and Methods," a modification of the Chapman mitis-salivarius medium proved to be most satisfactory for the cultivation of enterococci on the filters. This modified medium was prepared as follows:

	Percent composition
Tryptone	2.0
Proteose peptone No. 3 (Difco)	1.0
Proteose peptone (Difco)	1.0
Glucose	.2
Sucrose	10.0
Dipotassium phosphate	.4
Sodium azide	.04

Final pH 7.0–7.2.

Sterilize at 121°·C. for 15 minutes.

Prepare a 1-percent 2,3,5 triphenyl tetrazolium chloride (TTC) solution, sterilize, and add aseptically to above medium just before use to give 0.01 percent final concentration.

After preparation, the base medium was used within a 1-week period.

When this medium is used, the enterococci develop on the filters either as flat colonies, which are light pink in color, or as raised, glistening colonies which are dark red with a pink periphery. At the end of 48 hours' incubation of the cultures at 35° to 37° C., the colonies ranged in size from 0.5 to 2 millimeters in diameter. Although colonies could be detected after the cultures had been incubated for 24 hours, better growth was obtained when the incubation period was 48 hours.

On tests made with polluted river water, colorless colonies of gram-positive bacilli have occasionally developed on the filters. Preliminary tests have indicated that these organisms could be inhibited by the addition of 0.000012 percent ethyl violet to the medium, although concentrations of 0.00012 percent of the dye did not appear to inhibit the growth of the enterococci. However, since the gram-positive bacilli have appeared so rarely on tests made to date, ethyl violet has not been added routinely to our enterococcus medium. We have recently found that 4 percent tryptose and 1 percent yeast extract can be substituted for the proportions of tryptone, proteose peptone No. 3, and proteose peptone used.

When our medium was used in tests with pure cultures of *S. faecalis*, the colony counts on the filters were comparable to those obtained on tryptose glucose agar plates. As well as supporting good growth of enterococci, this medium proved to be highly selective and efficient for the cultivation of enterococci from contaminated water. Potassium tellurite and merthiolate were not suitable as selective agents since they also inhibit growth of the enterococci.

Efficiency for Enterococcus Counts

The efficiency of the membrane filter for the determination of numbers of enterococci in water, as used with our enterococcus medium, was compared with other procedures, particularly with the most probable number (MPN) methods described by Winter and Sandholzer (*3*) and by Litsky and his associates (*11*). Tests were made on water samples taken from a reservoir and six different rivers. The coliform density for these samples was also determined by the standard methods MPN and by membrane filter techniques, which are similar to those outlined by Kabler (*14*) and are described in a paper now in press (*15*). Examples of the results obtained with the comparative filter and MPN techniques for coliform density and the enumeration of enterococci are recorded in tables 1 and 2.

Table 1. Comparison of numbers of enterococci in water samples by membrane filter and Winter-Sandholzer techniques, and data on coliform densities

Sample No.	Enterococci		Coliforms	
	Filter technique	Winter-Sandholzer MPN technique	Filter technique	Standard 5-tube MPN technique
1	11	2	100	350
2	16	0	450	540
3	320	6. 8	160	110
4	224	4. 5	50	110
5	61	0	80	130
6	121	49	530	920
7	182	110	640	920
8	48	49	1, 520	1, 600
9	146	130	150	350

NOTE: Numbers indicate enterococci or coliforms per 100 ml. of water and represent an average of counts on duplicate Bac-T-Flex filters.

The membrane filter technique always gave higher counts for enterococci than did the Winter-Sandholzer method and gave higher counts than the procedures of Litsky and associates for all but three of the samples listed. Thus, when it was used in conjunction with the membrane filters, our selective medium proved highly efficient for the detection and enumeration of enterococci in the water samples. The colonies of enterococci were pink to red in color, and practically all other types of bacteria were inhibited even when 100 milliliters of highly polluted water was filtered through the membranes (see the photograph).

Table 2. Comparison of numbers of enterococci in water samples, by membrane filter and Litsky-Mallmann-Fifield techniques, and data on coliform densities

Sample No.	Enterococci		Coliforms	
	Filter technique	Litsky-Mallmann-Fifield MPN technique	Filter technique	Standard 5-tube MPN technique
1	4	0	140	220
2	2	2	690	1, 600
3	6	0	350	145
4	48	0	480	1, 600
5	48	0	430	350
6	98	79	100	130
7	0	0	30	23
8	6	2	500	145
9	5	2	184	170
10	242	220	360	920
11	27	0	680	920
12	205	170	1, 530	1, 600
13	433	240	1, 560	1, 600
14	129	9. 4	1, 060	920
15	508	350	320	540
16	594	920	290	540
17	1, 196	1, 600	200	540
18	284	350	80	33

NOTE: Numbers indicate enterococci or coliforms per 100 ml. of water and represent an average of counts on duplicate Bac-T-Flex filters.

During the study, more than 300 of these pink and red colonies were isolated from the membranes used for the tests on water samples reported in tables 1 and 2. These colonies were first inoculated into the confirmatory ethyl violet azide broth of Litsky, Mallmann, and

Fifield, and then further tested for their ability to grow in 0.1 percent methylene blue milk; in a glucose broth containing 6.5 percent sodium chloride; in a glocuse broth adjusted to pH 9.6; and at a temperature of 45° C. All but six of the cultures so tested were identified as enterococci. These six cultures were isolated from red pinpoint colonies which were atypical and rough in appearance. For some of the water samples tested, all of the pink or red colonies which developed on a particular filter were cultured as outlined previously, and all proved to be enterococci.

In addition to its efficiency, the procedure, when compared with other techniques previously developed for the detection of these organisms, saves considerable time, labor, and materials.

Although the counts for enterococci were comparable on both types of membrane filters, the colonies could be counted more easily on the filters with the 8-mm.-square grid markings (*B*) than on those with the 3-mm.-square grid (*A*). Because the former filter is also the more flexible and durable of the two types, it was used for the majority of tests reported here. Also, the apparatus supplied by its manufacturer had the advantage of providing a larger filtration area on the surface of the membranes.

Although the numbers of coliforms were generally greater than the numbers of enterococci in the water samples tested, enterococci were detected in all samples containing coliforms except one. This was a sample of river water which had a low coliform index. The coliforms as determined by the filter and the standard methods MPN procedures were in good agreement. Further studies are necessary to establish the significance of the numbers of enterococci as compared with the significance of the numbers of coliform bacteria in the determination of the sanitary quality of water. However, it would appear that the medium and filter technique described in this paper provide a relatively simple and efficient method for the quantitative determination of enterococci in water or other materials. By use of this procedure, tests for these bacteria may thus prove to be more reliable for establishing the sanitary quality of water and foods than have tests for coliform organisms.

Summary

A highly selective and efficient medium has been developed for use with membrane filters in the detection of enterococci in water. With this membrane filter technique, the counts for enterococci were generally higher than those obtained by other procedures. The method affords a relatively simple and direct means for the determination of the numbers of enterococci in water or in other materials.

EQUIPMENT REFERENCES

(*A*) Millipore filters (3-mm.-square grid markings) and apparatus. Supplied by Lovell Chemical Company, Watertown, Mass.

(*B*) Bac-T-Flex filters (8-mm.-square grid markings), Coli 5 apparatus, and S&S absorbent pads No. 470. Supplied by Carl Schleicher & Schuell Co., Keene, N. H.

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East Coast Migrant Conference Report

Leaders from 10 east coast States and representatives of public and private groups working directly with migrant families met in Washington, D. C., during May 17–19, 1954, to work out steps that will lead to better health, schooling, and security for the children of migrants—and, to some degree, for their families. The movement of seasonal farm workers has long created problems, both for the migrants and for the communities where they live temporarily.

Conference participants explored many areas in which they thought action was needed. Health questions were concentrated on such subjects as health records, environmental sanitation and housing, and financing of health services. In the short time available, the conferees sketched out a general guide for action and for further exploration. Their ideas and specific proposals, together with individual reports from the States taking part, are contained in a 110-page report which is available upon request to Paul Blackwood, Room 3280, Department of Health, Education, and Welfare, Washington 25, D. C.

The conference was sponsored by agencies of the Department of Health, Education, and Welfare—the Public Health Service, the Children's Bureau, the Office of Education, and the Bureau of Public Assistance. The States represented were Delaware, Florida, Georgia, Maryland, New Jersey, New York, North Carolina, Pennsylvania, South Carolina, and Virginia.