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Enrichment Procedure for Use with the Membrane Filter for the Isolation and Enumeration of Fecal Streptococci in Water[1]

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ABSTRACT

ROSE, ROBERT E. (Massachusetts Department of Public Health, Amherst), AND WARREN LITSKY. Enrichment procedure for use with the membrane filter for isolation and enumeration of fecal streptococci in water. Appl. Microbiol. **13**:106–108. 1965.—By the use of PYC enrichment medium, the recovery of fecal streptococci from river water has been increased more than twofold over that of M-Enterococcus Agar. Of the isolates tested, 94.6% could be classified as enterococci or enterococcus biotypes. This method seems to yield a larger number of strains which would not normally be revealed. Serological typing of atypical streptococcus strains isolated indicates that the majority of these "biotypes" can be placed in the enterococcus group.

Following Sherman's (1937) comprehensive review of the streptococci, the use of the fecal streptococci as indicators of human and animal pollution in water has been advocated by a number of investigators (Mallman and Seligmann, 1950; Litsky, Mallman, and Fifield, 1953; Slanetz and Bartley, 1957; Kjellander, 1960). In the past decade, the formulation of media for the recovery of streptococci by the membrane-filter method has given even greater impetus to the importance of these organisms. With this technique, a direct count may be obtained efficiently and economically. Likewise, it is possible to examine larger quantities of water than were practical by the most probable number (MNP) tube or plate count methods.

During a routine survey on an indoor swimming pool, minute streptococcal-like colonies were observed growing on M-Enterococcus Agar (Difco). When these colonies were transferred to Brain Heart Infusion (BHI) Broth (Difco) for subsequent identification, no growth could be noted. This indicated that the M-Enterococcus Agar was either incomplete or inhibitory for the primary isolation of all of the enterococci in the sample, or it had permitted minute nonenterococcal colonies to develop. The work reported herein was an attempt to clarify this situation by formulating a more efficient medium for the recovery and isolation of the enterococci from water by use of the membrane-filter technique.

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MATERIALS AND METHODS

PYC (peptone-yeast extract-Casitone) enrichment broth was formulated on the basis of its efficiency and comparative results for enterococci recovery. The formula for PYC enrichment broth is as follows: nutrient broth (Difco), 0.8%; Proteose Peptone (Difco), 0.2%; Proteose Peptone #3 (Difco), 2.0%; yeast extract (Difco), 0.6%; Casitone (Difco), 0.2%; distilled water, 100 ml; final *p*H, 7.0 to 7.1.

The prepared broth was dispensed into screw-capped test tubes (the caps supplied with rubber liners) and autoclaved at 121 C for 10 min. After sterilization, the test tubes were allowed to cool at room temperature, and then stored at 10 C prior to use.

Essentially, the recovery procedure followed was (i) an enrichment phase in PYC broth followed by (ii) an incubation phase on M-Enterococcus Agar. To determine the efficiency of the PYC enrichment procedure, split samples were analyzed with only M-Enterococcus Agar for comparison. M-Enterococcus Agar (4 to 5 ml) was poured into 60-mm plastic petri dishes and allowed to solidify. After this agar had hardened, a filter pad was placed in the upper half of the inverted petri dish and saturated with 1.8 ml of PYC enrichment broth. The water samples were filtered through the membrane filter. (All equipment for use with the membrane-filter technique was obtained from the Millipore Filter Corp., Bedford,

TABLE 1. *Comparison of recovery* of enterococci by use of M-Enterococcus Agar with and without enrichment*

Sample no.	Colonies per 100 ml on M-Enterococcus Agar	Colonies per 100 ml on M-Enterococcus Agar with enrichment
1	850	1,900
2	390	700
3	165	350
4	1,180	1,592
5	280	865
6	165	300
7	640	883
8	280	730
9	750	750
10†	6.6×10^4	7.6×10^5
11	300	1,580
12	400	1,430
13	1,160	3,130
14	730	3,100
15	280	1,380
16	86	140
17	180	350
18	80	130
19	192	312
20†	9,500	12,400
Arithmetic mean	445	1,090

* Recovery ratio of PYC–M-Enterococcus Agar was 2.44:1.

† Sewage samples.

TABLE 2. *Total numbers of enterococcus and nonenterococcus strains recovered**

Species	No. of strains	Per cent of total
Enterococcus	376	81
Enterococcus biotypes	63	13.6
Nonenterococcus species	25	5.4

* The total number of strains examined was 464. Total enterococcus strains recovered was 439. The per cent recovery was 94.6.

Mass.) The volume of the sample used depended on the turbidity and degree of pollution of the water. The membrane was then placed on the saturated enrichment pad and incubated for a period of 3 to 18 hr. After the enrichment procedure, the membrane was transferred to the agar substrate and incubated at 35 C in an inverted position for the remainder of the 48-hr incubation period. Split, controlled samples with M-Enterococcus Agar were also incubated for 48 hr. All of the determinations were carried out in triplicate, and all plates were examined at 10× magnification under a Spencer AO Cycloscopic Microscope.

All red or pink colonies visible at this magnification were counted and presumptively designated as fecal streptococci. The average count of the three plates based on 100 ml of sample was reported. Whenever possible, all of the colonies were picked after 48 hr of incubation and subjected to the following classification scheme using BHI as the basal medium: growth at 45 C (48 hr); growth at 10 C (5 days); growth in 6.5% NaCl (48 hr); growth in 40% bile (48 hr); reduction of potassium tellurite 1:2,500 (5 days); the appearance of gram-positive cocci with or without pleomorphism and absence of catalase activity.

Several of the isolated strains that did not conform to the classical classification were serologically typed by the Lancefield (1933) precipitin technique with the autoclave method of Rantz and Randall (1955).

TABLE 3. *Comparison of enrichment time with PYC broth*

Sample no.	M-Enterococcus Agar (48 hr)	PYC enrichment broth	
		3 hr	18 hr
1	170*	205*	295*
2	155	210	165
3	600	17,700	15,200
4	1,500	1,850	1,700

* All counts based on 100-ml sample.

RESULTS AND DISCUSSION

Eighteen river and two sewage samples were examined for the presence of fecal streptococci. The comparative results of these analyses are tabulated in Table 1. Samples 10 and 20 were obtained from settled sewage and a trickling filter effluent, respectively, and were not included in the calculation of the mean which represented the river samples. It was noted that samples 4, 7, and 9 showed little increase in recovery when the enrichment procedure was used. An investigation of the sampling source revealed that dye-containing industrial waste was being discharged into the river just above the sampling point, and it is assumed that this water had a toxic effect on those organisms; thus, higher recovery, even with enrichment, was impossible.

The arithmetic mean for the 18 river samples was calculated as 445 per 100 ml for M-Enterococcus Agar, and 1,090 per 100 ml when the enrichment was used. The recovery ratio of M-Enterococcus Agar to enrichment was 1:2.44, a notable increase with enrichment.

A total of 464 colonies was picked and subjected to the aforementioned tests. Of these, 376 were classified as members of the enterococcus group, 63 as enterococcus biotypes (Kenner, Clark, and Kabler, 1960), and 25 as nonstrepto-

coccal species as ascertained by positive catalase activity (Table 2). Of the biotype strains, 36 were picked at random, and all were found to contain the group D polysaccharide which is common to the enterococcus group.

In the examination of fresh feces, Kenner et al. (1960) found 19.1% atypical streptococci species which they termed "biotypes." In the work presented here, there were 13.6% of these "biotypes" isolated from river water. Because these organisms have been shown to be present in fecal matter, and the group D polysaccharide has been demonstrated in 36 strains isolated from water, it would appear that: (i) these organisms can be given fecal indicator status, and (ii) the enrichment procedure is sensitive to the recovery of total fecal streptococci in a water sample.

To determine the optimal enrichment incubation, samples were split into three portions. Each sample was subjected to a 3- and 18-hr enrichment together with a nonenrichment control. Following the enrichment incubation, the filters were placed on M-Enterococcus Agar and incubated for the remainder of the 48-hr period as indicated above. It was concluded that though enrichment procedures yielded higher counts than the control, there was no appreciable difference between the 3- and 18-hr incubation used in the enrichment technique (Table 3). It is our opinion that a 3-hr enrichment incubation should be used routinely for this technique. However, should a water sample arrive at the laboratory late in the day, the above data suggest that the membrane may be allowed to remain on the enrichment pad until the next morning without any appreciable loss in viability.

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