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Literature Cover Sheet

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Title: Membrane Filter Technique for Enumeration of Enterococci in Maine Water

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Topic: Evaluation of the mE procedure Vs KF method for enumeration of Enterococci

Highlights:

- This study reported a false positive rate of 10% and a false negative rate of 12% for the mE membrane filtration method after verifications.

* see page 66

Membrane Filter Technique for Enumeration of Enterococci in Marine Waters

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A membrane filter procedure is described for the enumeration of enterococci in marine waters. The procedure utilizes a highly selective and somewhat differential primary isolation medium followed by an in situ substrate test for identifying colonies of those organisms capable of hydrolyzing esculin. The procedure (mE) was evaluated with known streptococci strains and field samples with regard to its accuracy, sensitivity, selectivity, specificity, precision, and comparability to existing methods. Essentially quantitative recovery was obtained with seawater-stressed cells of *Streptococcus faecalis* and *S. faecium*. Neither *S. bovis*, *S. equinus*, *S. mitis*, nor *S. salivarius* grew on the medium. The selectivity of the medium was such that a 10,000-fold reduction in background organisms was obtained relative to a medium which contained no inhibitors and was incubated at 35 C. About 90% of those typical colonies designated as enterococci confirmed as such and about 12% of the colonies not so designated were, in fact, identified as enterococci. Plate to plate variability across samples approximated that expected by chance alone. Verified recoveries of enterococci from natural samples by the mE procedure, on the average, exceeded those by the KF method by one order of magnitude.

Shortly after J. P. Laws and F. W. Andrews first reported streptococci from the gastrointestinal tract, Houston noted that these organisms appeared to be characteristic of sewage and animal fecal wastes. He suggested that they were indicative of dangerous pollution because they are readily demonstrable in recently polluted waters and seemingly absent from waters above suspicion of contamination (18). Since that time, a volume of data has been accumulated categorically demonstrating that fecal streptococci are associated with the fecal wastes of man and lower animals and that they can be isolated from polluted water containing such wastes (14, 20). Nevertheless, this group of organisms has not been generally accepted as an indicator of fecal contamination for at least two reasons. First, coliforms and coliform biotypes have been a more attractive means of identifying fecal contamination because early workers found them easier to quantify and they are present in larger numbers in feces, sewage, and polluted waters. Second, there has been a good deal of confusion concerning the identity of the fecal streptococci, particularly as it relates to their ecological distribution. This is reflected in the fact that those streptococci which can be found in the feces of man and other warm-blooded

animals have been referred to variously as enterococci, fecal streptococci and, more recently, group D streptococci. The composition of these three groups is shown in Fig. 1. It can be seen that two organisms not associated with humans, *Streptococcus bovis* and *S. equinus*, and two organisms associated with humans, but not exclusively with fecal wastes, *S. mitis* and *S. salivarius*, are included in one or more of the groups. Ideally, the indicator should be limited to the fewest number of species or biotypes which are most closely or exclusively associated with the fecal wastes of man, i.e., *S. faecalis* and *S. faecium*. Facile methods to do this have not been available. Hence, broader groups of streptococci have been used at times, because these were the biotypes recovered by the procedures available.

The taxonomy and distribution of this group of organisms has been reviewed by Hartman et al. (8), Kjellander (11), and revised recently in *Bergey's Manual for Determinative Bacteriology*, 8th ed. (2).

Early attempts to quantify fecal streptococci relied on enrichment tube procedures associated with the use of the most probable number method. In 1940 Mallmann (15) suggested the use of azide lactose broth. This was later

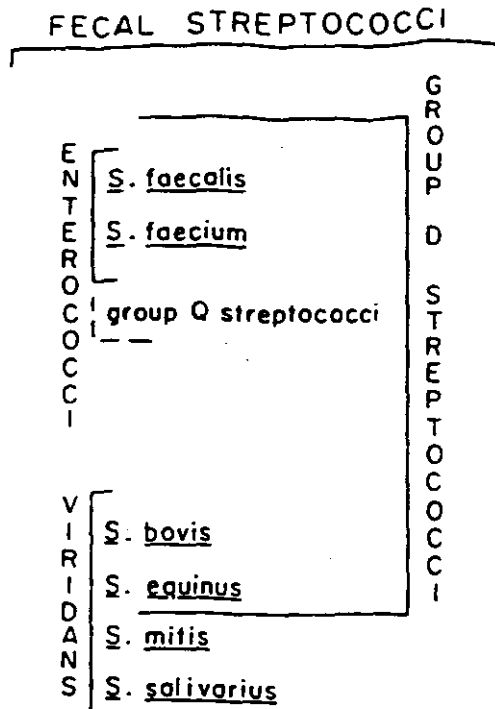


FIG. 1. Distribution of streptococci species among three groups. This study was completed prior to the release of the 8th edition of *Bergey's Manual for Determinative Bacteriology* (2), which has classified *S. zymogenes* as *S. faecalis*, subspecies *zymogenes* and *S. durans* as equivalent to *S. faecium*.

modified by Mallmann and Seligmann (16) using Roth's basic azide dextrose medium, followed by a confirmation in ethyl violet azide broth as described by Litsky et al. (13). This procedure has been evaluated in marine waters (3). Hajna and Perry (7) developed the SF medium; and Winter and Sandholzer (23) described presumptive and confirmatory media which use sodium azide and/or high concentrations of NaCl as inhibitors.

The quantification of enterococci by a membrane filter procedure was first reported by Slanetz and Bartley in 1957 (21); and, in 1961, Kenner et al. (10) described the KF method for the enumeration of fecal streptococci. In 1966 Isenberg et al. (9) reported a plating procedure (PSE) for the quantification of group D streptococci. This medium offered increased recoveries and easier differentiation based on the use of the esculin reaction. The recoveries by these and other procedures have been compared by a number of workers (19, 22).

Impetus for the present investigation devel-

oped from a comparison of the KF methods and PSE medium (used in conjunction with membrane filtration) for the isolation of fecal streptococci from marine waters at beaches in the vicinity of New York City. In this preliminary investigation, it was observed that the confirmed recoveries of fecal streptococci by the modified PSE procedure exceeded those by the KF method by about one order of magnitude. However, the modified PSE procedure as used was deficient in that overgrowth by background microorganisms was a serious problem. The present report describes the evaluation of a procedure for the enumeration of enterococci designed to obviate the problems noted above. In addition, an attempt was made to make the method more specific, i.e., to eliminate the recovery of the viridans group (*S. mitis*, *S. salivarius*) and the two organisms exclusively associated with animal feces (*S. equinus* and *S. bovis*). The method (mE) was evaluated against the following criteria: (i) accuracy, recovery of at least 75% of the viable *S. faecalis* and *S. faecium* cells following a stress imposed by exposure to sea water for 48 h at 4°C; (ii) selectivity, the reduction of background organisms in naturally polluted waters by at least three orders of magnitude (1,000-fold); (iii) specificity, colonies designated as positive should verify as such at least 75% of the time, and no more than 10% of those designated as negative should verify as enterococci; (iv) precision, with field samples, the D^2 (4) value distribution approximates that estimated by chance; and (v) comparability, the accuracy and sensitivity of the method be equal to or greater than existing membrane filter methods.

MATERIALS AND METHODS

Cultures and field samples. The recovery and accuracy studies were performed with cultures of *S. faecalis*, *S. faecium*, *S. bovis*, *S. equinus*, *S. mitis*, and *S. salivarius* provided by R. Facklam (Center for Disease Control, Atlanta, Ga.), and with a strain of *S. faecalis* isolated from New York Harbor. Suspensions of these organisms were prepared from brain-heart infusion broth (BHI, Difco) cultures incubated at 37°C for 20 h. After incubation, the organisms were washed three times in sterile phosphate-buffered saline (NaH_2PO_4 , 0.58 g; Na_2HPO_4 , 0.25 g; NaCl, 0.95 g; distilled water, 100 ml). Aliquots of the resultant suspensions were delivered into flasks of sea water passed through a 0.2- μm membrane filter. These were held at 4°C and sampled periodically by the mE method and a control procedure (spread plates on BHI agar) to determine the number of recoverable organisms.

Field samples were collected from marine and

estuarine waters in sterile containers held at 4 to 6 C and assayed within 12 h of collection.

Membrane filtration. Appropriate volumes of the test suspensions or water samples used in evaluating the experimental medium were passed through membrane filters (47-mm diameter, 0.45- μ m pore size). When the portion of the water sample to be filtered was less than 20 ml, it was brought to at least that volume with phosphate-buffered saline to wash residual organisms onto the membrane. The glass filter holders were sterilized for 2 min in an ultraviolet sterilizing apparatus (Millipore Filter Corp.). The membrane filters were obtained presterilized from the manufacturer.

Recovery media: mE. The formulae and methods of preparation of mE medium and the esculin-iron agar substrate used in the *in situ* esculin test are given in Table 1. After a basal medium containing peptone, sodium chloride, yeast extract, esculin, and ferric ammonium citrate was shown to quantitatively recover the organisms, a search for appropriate inhibitors was initiated. A number of candidates were screened for this purpose initially by using a modification of the gradient plate method of Szybalski as described by Levin and Cabelli (12). Nalidixic acid and sodium azide are used to inhibit gram-negative organisms and actidione to inhibit fungi. Triphenyl tetrazolium chloride in the concentration used colors the colonies, differentiates enterococci from other streptococci based upon its reduction, and has a slight inhibitory effect on some background microorganisms. Esculin is included to induce the enzyme catalyzing its hydrolysis. The mE plates are incubated for 48 h at 41 C; the elevated incubation temperature also inhibits some of the indigenous microbial flora.

In situ esculin substrate. The hydrolysis of esculin is used in the characterization of enterococci. Initially, the esculin indicator system was included in

the primary medium. This resulted in dark red colonies approximately 2 mm in diameter with black halos in the medium resulting from the reaction of cumarin with the ferric chloride. When more than 20 colonies were present, however, the zones coalesced, making it impossible to determine which of the colonies was positive. This problem was overcome by the use of an *in situ* test in which, after incubation, the membrane is transferred to an esculin-iron agar plate (Table 1). After 20 to 30 min at 41 C, small black spots appear under the positive colonies, permitting enumeration of at least 80 enterococcus colonies per plate without problems of coalescence.

Control media. KF (Difco) and PSE (Pfizer) media were prepared and used following instructions from the manufacturers and *Standard Methods for the Examination of Water and Wastewater* (1). As noted earlier, the PSE medium was used in a membrane filter procedure.

Verification of colonies. Verification of colonies as enterococci was accomplished by using the bile-esculin medium of Schwan in combination with a modification of the procedure of Facklam and Moody (5), i.e., (i) growth at 45 C in BHI broth; (ii) a negative catalase test; (iii) esculin hydrolysis; (iv) growth on 40% bile-blood agar; (v) an acid reaction in litmus milk; and (vi) a positive Gram stain.

RESULTS

The accuracy of the mE method was determined by comparing the recoveries obtained by this procedure to those observed when eight species of streptococci were spread plated on BHI agar. The suspensions, whose initial cell densities varied between 10^4 to 10^8 per ml, were prepared in filtered estuarine water and held at 5 C for periods up to 9 days. The average relative recovery of *S. faecalis* and *S. faecium* over the 9-day period was 102%; *S. bovis*, *S. equinus*, *S. mitis*, and *S. salivarius* recoveries were 0.0001% or less (Table 2).

The selectivity of the mE method was such that a 10,000-fold reduction in background organisms was obtained relative to that observed when the inhibitors were omitted from mE medium and the plates were incubated at 35 C. This 99.99% reduction was obtained with samples whose initial background densities were 10^4 to 10^8 cells per 100 ml.

The specificity of the mE procedure was examined by determining (i) the percentage of typical colonies which did not verify as members of the enterococci group (false positives) and (ii) the percentage of other colonies, those which did not possess the typical colonial characteristics which, in fact, were enterococci (false negatives). The 2,231 colonies examined were isolates from polluted marine and estuarine water samples collected at six locations along the east coast of the United States. Most of the

TABLE 1. Preparation of mSD medium and EIA substrate

mSD		EIA	
Ingredients*	g/liter	Ingredients*	g/liter
Agar	15.0	Agar	15
Peptone	10.0	Esculin	1.0
NaCl	15.0	Ferric citrate	0.5
Esculin	1.0	Distilled water	1,000 ml
Yeast extract	30.0		
Actidione	0.050		
Sodium azide	0.150		
Distilled water	1,000 ml		

* Autoclave at 121 C for 15 min. After autoclaving, add nalidixic acid, 0.24 g; and triphenyl tetrazolium chloride, 0.15 g. Adjust pH to 7.1 ± 0.1 and pour in 3.5-ml amounts to 50-mm membrane filter plates.

* Adjust pH to 7.1 ± 0.1 before autoclaving at 121 C for 15 min. Pour in 3.5-ml amounts to 50-mm membrane filter plates.

TABLE 2. The recovery of stressed fecal streptococci on mSD medium

Organism	% Recovery* after exposure (days)				
	0	1	3	6	9
<i>S. faecalis</i>	107	ND ^a	94	123	87
<i>S. faecium</i>	102	ND	126	73	109
<i>S. faecium</i> ^c	114	ND	108	46	100
<i>S. faecalis</i> subsp. <i>zymogenes</i>	117	ND	118	ND	113
<i>S. bovis</i>	0.001	<0.0001	ND	ND	ND
<i>S. equinus</i>	<0.0001	ND	ND	ND	ND
<i>S. salivarius</i>	<0.0001	ND	ND	ND	ND
<i>S. mitis</i>	<0.0001	ND	ND	ND	ND

* Suspensions held in seawater at 5 C for number of days as indicated.

^a Relative to recovery on BHI spread plates.

^c Obtained in 1973 from R. Facklam as *S. durans*.

^d No data.

isolates came from samples collected at beaches in the vicinity of New York City. In general, all the colonies on a given plate were examined. Ninety percent of the typical colonies and 11.7% of the other colonies were enterococci (Table 3). Neither *S. bovis*, *S. equinus*, *S. mitis*, nor *S. salivarius* were encountered.

The precision of the mE method was determined from D^2 values for assay variability calculated from the following equation as given by Eisenhart and Wilson (4): $D^2 = N \sum X_i^2 - (\sum X_i)^2 / \sum X_i$, where $\sum X_i$ is the summation of the plate counts X_1, X_2, \dots, X_n , and N (the number of replicate plates per sample) was 5. The D^2 values calculated from the examination of 15 polluted, marine, and estuarine water samples are displayed in Fig. 2 along with the expected D^2 control limits for $P = 0.005, 0.025$ and 0.5 . In the event of excessive variability among the five replicate determinations (plates), hence, poor reliability of a single determination, the observed D^2 values should have exceeded the control limits more frequently than expected by chance alone. By chance alone, one D^2 value in 40 would have been expected to exceed the $P = 0.005$ limit. However, it can be seen that none of the values exceed the limits, and that they are evenly distributed around the $P = 0.5$ limit (Fig. 2).

Sixteen water samples collected from a variety of sources were assayed in parallel by the KF, PSE, and mE methods. Typical colonies as described for the various procedures were verified as stated in Materials and Methods. Thus, the data presented are derived from verified recoveries. As can be seen from Table 4, neither

the PSE nor KF recoveries approached those obtained with the mE method, although those by the PSE and KF methods did approximate each other. The average number of colonies on the filters in these 13 trials was 70, ranging from 9 to 110. In a second, more extensive set of trials, fecal streptococcus densities obtained by the KF method were compared

TABLE 3. Specificity of the mSD procedure for the enumeration of group D streptococci

General sample location	No. of colonies and % verification			
	Typical colonies		Other colonies	
	No. examined	% False positive	No. examined	% False negative
Conny Island, N.Y.	1,225	11	107	18
Riis Park, N.Y.	577	10	104	11
Miami, Fla.	16	0 ^a	16	0 ^a
Boston Harb., Mass.	59	3.0	ND ^b	ND
Connecticut	67	4.0	20	0 ^a
Rhode Island	30	0 ^a	10	0 ^a
Overall	1,974	10.0	257	11.7

^a No false-negative or positive colonies.

^b No data.

D^2 VALUES FOR mSD AGAR

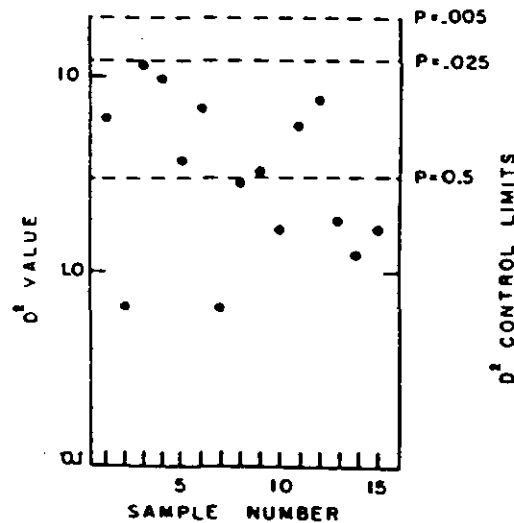


FIG. 2. Precision of the mSD procedure for enterococci as extended from dispersion of D^2 values. Symbols: (●) D^2 value calculated from five replicate plates at each point. ----: control limits when probability is as stated.

TABLE 4. Comparison of verified recoveries of group *D* streptococci by the mSD, PSE and KF methods

Location	Trial	mE recovery (colonies/plate)	% (mSD) recoveries* by	
			PSE	KF
Providence River	1	45	82	62
	2	23	70	74
	3	90	68	58
	4	55	78	94
	5	70	49	73
Coney Island	1	110	59	61
	2	82	60	77
	3	21	48	67
Boston Harbor	1	89	88	75
	2	97	59	70
	3	73	47	73
Miami	1	9	ND*	66
	2	107	ND*	33
Stonington Harbor	1	9	39	75
New London Harbor	1	61	25	38
Mystic Harbor	1	85	68	9
Avg		70	60	63

* Values obtained from the average of triplicate plate counts.

* No data because the PSE plates were overgrown with background organisms.

directly to those by the mE procedure (Table 5). In only three instances did the KF recoveries exceed the mE recoveries; on the average, the mE recoveries were about 10 times greater than those by the KF procedure. The samples reported in Table 4 were obtained during the winter months and those reported in Table 5 were obtained in July and August.

DISCUSSION

The mE procedure has satisfied most of the predetermined criteria for a primary, selective-differential method for the enumeration of enterococci in marine waters. The only exception was the rate of false-negative colonies.

Essentially quantitative recovery of *S. faecalis* and *S. faecium* was obtained with unstressed and stressed cells. Although this approach is basically artificial in that the work was done with pure cultures rather than natural samples, it is not logistically feasible to perform such experiments with natural samples. *S. bovis* and *S. equinus* were not recovered in significant numbers of mE medium, nor were they isolated from natural samples. Therefore, it may be assumed that these two species, whose origin is animal feces, are not included in

densities of enterococci as obtained by the mE procedure. Relative to other methods, this increases the specificity of the mE procedure for enterococci of human origin. However, *S. faecalis* and *S. faecium* do occur in animal feces (2). In addition, biotypes of *S. faecalis* have been reported (4, 15) as being associated with vegetation unpolluted with human fecal wastes and with insects. The former are differentiated by their ability to hydrolyze starch, a characteristic which could be performed as a subsequent *in situ* test.

The selectivity criterion for the evaluation of mE methods assumes that the marine recreational waters to be examined for enterococci generally would contain less than 10^3 background organisms per ml (those bacteria which grow in 48 h on the mE medium when the inhibitors are omitted and the plates are incubated at 35 C). The required 1,000-fold (99.9%) reduction in the level of these organisms, to be achieved by the combination of inhibitors and

TABLE 5. Comparison of verified recoveries of fecal streptococci and other organisms by the mSD and KF methods

Location	Trial	Recovery per 100 ml by group and method			
		Fecal streptococci		Other colonies	
		mSD	KF	mSD	KF
Coney Island, N.Y.	1	38	<1	54	13
	2	38	ND*	35	TN*
	3	270	6	190	<1
	4	660	ND	TN	TN
	5	670	ND	240	TN
	6	310	20	430	<10
	7	70	4	123	<1
	8	7	1	4	<1
	9	70	4	70	<1
	10	1,510	6	310	<1
Rockaways, N.Y.	1	170	12	940	<1
	2	230	17	1,090	<1
	3	440	8	TN	<1
	4	51	5	450	<1
	5	25	61	56	<10
	6	49	62	24	<10
	7	31	2	<1	<1
	8	16	8	127	<1
	9	32	5	100	<1
	10	47	2	53	<1
	11	36	8	2	<1
	12	12	57	89	<1

* No data, confluent growth of background organisms.

* Too numerous to count.

the elevated incubation temperature, would result in 50 colonies per filter when a 50-ml water sample is assayed. The differential characteristics of the medium then should permit the detection of a single group enterococcus colony on such a filter. The actual reduction (99.99%) exceeded this requirement, thereby permitting the detection of group D streptococci in the presence of 500,000 background organisms deposited on the filter.

Although the rate of false-negative colonies (11.7%) slightly exceeded the specificity criterion (10%), the false-positive rate (10%) was markedly less than the specified limit (25%). Therefore, it may be assumed that verification of a number of typical and other colonies is necessary only when an operator is being trained. In the absence of verification, estimates of enterococci densities should be designated as presumptive. After verification, the estimates would be considered as confirmed.

The results demonstrate (Fig. 2) the precision (reproducibility) of the mE procedure. The plate to plate variability over the samples examined was that expected by chance alone; that is, the D^2 estimates distributed equally on both sides of the $P = 0.5$ limit of 3.2 and none exceeded the $P = 0.025$ limit.

The recovery efficiency of the mE method is comparable to, or better than, that by the KF method for the examination of enterococci in estuarine waters; in addition, it provides higher confirmed recoveries. The difference between recovery ratios (mE/KF) observed at Coney Island in the summer months (1.5; Table 4) and winter months (54; Table 5) may be a function of a seasonal variation (water temperature, rainfall). As noted previously, the method measures a more specific portion of the fecal streptococcus population and one that appears to be a close association with the fecal wastes of humans.

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