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Improved Membrane Filter Method for Fecal Coliform Analysis

ROBERT E. ROSE, EDWIN E. GELDREICH, AND WARREN LITSKY*

*Millipore Corporation, Bedford, Massachusetts 01730, National Environmental Research Center, Environmental Protection Agency, Cincinnati, Ohio 45286, and Department of Environmental Sciences, University of Massachusetts, Amherst, Massachusetts 01002**

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A two-layer agar method has been developed which consistently yields higher recovery of fecal coliforms on membrane filters when compared to the existing membrane fecal coliform procedure. This method has been evaluated by three laboratories using samples of raw and chlorinated waste water, and reservoir, river, and marine waters. Verification of 1,013 fecal coliform colonies isolated from 61 water samples averaged 92% on this proposed procedure. Comparison with the Standard Methods membrane fecal coliform procedure revealed the two-layer agar method had an overall increased sensitivity to fecal coliform detection in these waters. It is therefore proposed that this procedure be evaluated as an alternative to the Standard Methods fecal coliform membrane Filter test in the examination of chlorinated secondary effluents, marine waters, and any natural waters that may contain pollutants with heavy metal ions.

Since the 13th edition of Standard Methods (1), the membrane filter (MF) has been accepted as an alternate method for the detection and enumeration of fecal coliform organisms in water. However, its application has been disputed for the examination of chlorinated waste water effluents. In light of recent criticisms of the membrane fecal coliform (m-FC) technique (R. A. Greene, presented at a branch meeting of Am. Soc. Microbiol., South Bend, Ind., 19 October 1973; R. A. Greene, R. H. Bordner, and R. V. Scarpino, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, p. 34; 7), some of the modifications that have been suggested to improve the test have been reviewed, and from this the two-layer agar method was proposed, developed, and evaluated.

J. E. Delaney, J. A. McCarthy, and R. J. Grasso (presented at the annual meeting of Am. Public Health Assoc., Detroit, Mich., 14 November 1961) developed a two-step recovery method which was claimed to be an efficient test for *Escherichia coli* type 1. The membrane was first incubated on tryptone bile agar at 44.5 C for 20 to 24 h and transferred to an absorbent pad saturated with a solution consisting of dimethyl amino benzaldehyde and oxone. Colonies exhibiting a pink to red color, due to the formation of indol, were considered to be *E. coli* type 1. This method was not widely accepted because of: (i) the necessity of transfer of the filter to a second medium, (ii) the fact that recovery is limited to only one member of the fecal coliform group, and (iii) the fact that toxicity of indol reagent nullified any further verification of these colonies.

In 1965, Geldreich et al. (5) published an MF method for the detection of fecal coliforms using a lactose broth base to which was added an indicator system of aniline blue and rosolic acid. This m-FC medium used at an incubation temperature of 44.5 C ± 0.2 C for 24 h is currently accepted for fecal coliform recovery with the MF.

The attempts by Delaney et al. and Geldreich et al. to develop a precise MF test were prompted by the inherent limitations of the most-probable-number (MPN) method, which include low precision and excessive time necessary for analysis. Despite these limitations, it can be advocated that the presumptive enrichment step of the MPN may increase recovery of coliforms at elevated temperatures by allowing for repair and minimum stress.

Using an enrichment procedure developed by Goetz (6) in 1953, McKee and McLaughlin (9) demonstrated that total coliform recoveries from settled sewage on MF were comparable with the standard MPN test. Later that same year McKee et al. (10) reported that total coliform recovery from chlorinated sewage was consistently lower when enumerated by MF than by the MPN technique. They concluded that this was due to the neutralization of the bactericidal effects of monochloramine in the presumptive broth tube but not on the MF.

Recently, an enrichment method of McCarthy et al. (8) was used by Lin (7) to determine whether coliform recoveries from chlorinated secondary effluents were comparable to MPN values when the two tests were done in parallel. Total and fecal coliform enumeration methods were evaluated. Lin concluded that the direct incubation at 44.5 C on m-FC medium was less efficient than the confirmed elevated coliform (EC) medium multiple-tube procedure for fecal coliforms, although the LES enrichment method for total coliform recovery on the MF appeared to be comparable to the completed multiple-tube method used for this same indicator group (8).

Using chlorinated effluent samples from five municipal treatment plants, Greene et al. (Abstr. Annu. Meet. Am. Soc. Microbiol., 1974, p. 34) compared total and fecal coliform recoveries by MF and MPN methods and confirmed the work of Lin. His initial results, employing the one-step m-Endo method, revealed lower recovery of total coliform than did the two-step LES method. To achieve better agreement between the fecal coliform tests he preincubated the membranes on pads saturated with dilute m-FC broth at 25 C for 2 to 6 h. The membranes were then transferred to pads saturated with full strength m-FC broth and incubated at 44.5 C for 25 h. This increased the recovery rate of fecal coliform, which prompted Greene to conclude that the standard m-FC procedure could not be applicable for chlorinated waste water effluents. Moreover, he suggested that there be further evaluation of methods which would afford maximum recovery of fecal coliform.

A. P. Stevens, R. J. Grasso, and J. E. Delaney (presented at the 7th Nat. Shellfish Workshop, New Orleans, La., June 1974) assessed the fecal coliform densities in estuary water using a pre-enrichment MF technique. They were of the opinion that the initial shock at 44.5 C adversely affected reproduction of metabolically injured cells. After a series of preliminary tests, a two-step procedure was proposed in which the MF was placed on LES holding agar consisting of bile salts and simple carbohydrates, incubated in a water bath at 35 C for 16 to 24 h, and then transferred to m-FC medium for the final 24-h incubation at 44.5 C. The recovery efficiency for this 48-h method was in the 90% range when compared to the MPN technique.

In addition to the high recoveries afforded by this procedure, the authors introduced a colony counting procedure that was based on colony morphology rather than color. Employing a dissecting microscope at 10 to 20× magnification equipped with a light source perpendicular to the plane of the filter, all blue colonies having a crystalline deposit on the surface were designated and counted as fecal coliforms. Coliform colonies of the nonfecal variety appeared blue but lacked the crystal appearance which is probably precipitated bile.

These findings suggest that the existing methodology (m-FC) for recovery of fecal coliform on membrane filters is less than ideal, especially from samples where these bacteria may have been metabolically injured, which may be the case in chlorinated sewage effluent. Furthermore, there is evidence which indicates that chlorine inactivation of some coliform cells may be reversed if the cells are allowed to repair prior to heat shock (10).

Considering the present definition of coliform bacteria, it is generally recommended that any medium designed for the recovery of these microorganisms should contain lactose and that the primary enrichment medium should not contain materials which may restrict the growth of these bacteria. Upholding these two tenets, a method for isolation and enumeration of fecal coliforms has been developed and evaluated.

MATERIALS AND METHODS

Two-layer medium development. The base medium (Table 1) was prepared by adding 1.5 g of agar to 100 ml of m-FC broth (Difco or BBL) and heating to a boil. After cooling to 45 C, 5.0 ml was added to a 50-mm-diameter tight-fitting petri dish and allowed to solidify at room temperature (approximately 30 min). Two milliliters of lactose broth containing 1.5 g of agar per 100 ml was pipetted as a second layer onto the base agar. Since the ingredients of the two agar media will eventually diffuse into each other, it is suggested that the base m-FC agar be prepared in advance and the overlay added within 1 h prior to use. It was determined that the base medium may be

TABLE 1. *Formulation of the two-layer medium*

Medium	Amt
Differential medium (bottom layer)	
m-FC medium	3.7 g
Agar	1.5 g
Distilled water	100 ml
Resuscitation medium (top layer)[a]	
Beef extract	0.3 g
Peptone	0.5 g
Lactose	0.5 g
Agar	1.5 g
Distilled water	100 ml

[a] Resuscitation medium equals 1× lactose broth plus 1.5% agar.

poured into plates and held at refrigeration temperatures (10 C) for 3 days.

This two-layer technique was based on the hypothesis which suggested (i) the need of a repair phase prior to incubation at the elevated temperature and (ii) that the repair and elevated temperature growth phase be confined to a single petri dish to avoid unnecessary handling. After the MF was placed on the two-layer media, the plates were incubated at 35 C for 2 h which allowed for optimum growth and cell repair, after which the temperature was increased to 44.5 C for 22 to 24 h to attain the necessary selectivity.

A programmed water bath or a solid-state heat block may be used when all samples are processed at once. However, when samples are processed during different periods of the day, the use of two incubators is necessary. The time required for the temperature of the heat block to rise from 35 to 44.5 C was determined to be approximately 45 min.

To determine the efficiency of the proposed method, the number of blue colonies that developed on the two-layer plates was compared with the direct m-FC technique as described in Standard Methods (1). Water bath incubation of all cultures in the direct m-FC technique was held to the prescribed 24-h total time and a water bath temperature of 44.5 C. Colony counts were made with binocular scope employing 10 to 15× magnification and a fluorescent light source.

Verification of fecal coliforms isolated on the test medium was performed by subculturing each blue colony into phenol red lactose broth or lauryl tryptose broth for 24 to 48 h at 35 C. Tubes showing gas production within this period were again subcultured to EC broth and incubated in a waterbath for 24 h at 44.5 ± 0.2 C (1).

Samples were collected from diverse waters that included estuarine waters of Massachusetts, raw sewage and chlorinated sewage effluents from the Billerica, Mass., sewage-treatment plant, polluted stretches of the Merrimac, Fort, and Mill rivers, and samplings at varying depths of a raw water impoundment near Walton, Ky. The reservoir water samples were collected during a period of prolonged dry weather and following a significant storm water runoff into the impoundment. All bacteriological examinations of these waters were performed at one of three different laboratories located near the sampling sites: Millipore Filter Corporation Laboratory at Bedford, Mass.; University of Massachusetts, Department of Environmental Sciences Research Laboratory at Amherst; and the Environmental Protection Agency, Water Supply Research Laboratory, Cincinnati, Ohio. Three or five replicate portions were prepared for cultivation on both the two-layer experimental medium and the m-FC agar direct method as recommended in Standard Methods (1).

RESULTS AND DISCUSSION

Sixty-one water samples were analyzed in the evaluation of the two-layer method. The choice of samples used in this evaluation was oriented to those waters that might have attenuated fecal coliform populations resulting from contact with the marine environment, exposure to chlorination of sewage effluents, contact with heavy metal ions in chemically polluted fresh water, and to natural forces of self-purification induced in storage of impounded natural waters. The results of these comparisons are summarized in Table 2. The count of the lactose two-layer method was higher than the companion direct m-FC method in all but two samples examined from the water supply reservoir. In these two instances, the difference was less than 10%. Improved sensitivity for fecal coliform detection with the two-layer agar method was greatest (ratios above 3.0) in the analysis of all marine waters and chlorinated sewage effluents, four (22%) raw sewage samples, and three (21%) river samples. The two-layer agar method recovered almost twice the number of fecal coliform colonies detected by the direct m-FC method when all counts from the 61 water samples are totaled, i.e., 3,560 fecal coliform colonies on the two-layer agar versus 1,843 on the controls, an increase of 93%.

No attempts were made to compare the fecal coliform recovery on the two-layer agar with the multiple-tube (MPN) method. Because of the increased efficiency of the proposed method, it is believed that the MPN and MF counts will be in close agreement. Work is now underway to substantiate this.

A total of 1,013 typical blue colonies were picked from the two-layer plates and subcultured to lactose broth at 35 C. Those tubes showing gas production within 48 h were confirmed in EC broth at 44.5 C for 24 h. Of the 1,013 colonies picked, 930 produced gas at the elevated temperatures, for an average verification rate of 92% (Table 3).

Our observations support the work of Stevens et al. in that there appears to be a difference in the morphology of some of the blue colonies. However, both smooth blue and crystalline blue colonies developing on this medium should be counted. Many of the smooth colonies that were positive in lactose broth and confirmed at the elevated temperature in EC broth were found to be citrate positive and after further tests were classified as *Klebsiella* sp. Since there is evidence in the literature that *Klebsiella* strains are found in feces (4) and can be potentially pathogenic (2), their presence as smooth blue colonies isolated from water certainly cannot be ignored (3).

Likewise, it should be noted that preliminary observations suggest that the formation of granules in the crystalline-appearing colonies may

TABLE 2. *Comparison of the two-layer agar versus direct m-FC procedures for fecal coliform densities from various waters*

Source	m-FC count	Two-layer agar count	Ratio (two-layer agar/direct m-FC)	Source	m-FC count	Two-layer agar count	Ratio (two-layer agar/direct m-FC)
Raw sewage	57	103	1.8	Water runoff	86	95	1.1
	23	26	1.1		100	106	1.1
	32	72	2.3		140	170	1.2
	16	20	1.3		80	100	1.3
	16	26	1.6		95	125	1.3
	10	31	3.1		53	55	1.1
	4	11	2.8		130	160	1.2
	1	10	10.0				
	2	14	7.0	Merrimack River	4	25	6.3
	46	91	2.0		76	183	2.4
	5	12	2.4		4	25	6.3
	15	23	1.5		76	183	2.4
	24	33	1.4		23	53	2.3
	15	22	1.5		15	38	2.5
	25	42	1.7		8	56	7.0
	51	98	1.9				
	12	32	2.7	Fort River	11	21	1.9
	7	40	5.7		4	11	2.8
					7	10	1.4
Chlorinated sewage	6	228	38.0		9	16	1.8
	1	26	26.0		24	29	1.2
	26	127	4.9				
	5	19	3.8	Mill River	6	12	2.0
					22	60	2.7
Water supply reservoir (dry period)	11	10	0.9				
	17	30	1.8	Marine waters	3	12	4.0
	44	44	1.0		21	79	3.8
	10	13	1.3		3	12	4.0
	21	31	1.5		21	79	3.8
	94	89	0.9		3	16	5.3
	6	10	1.7		30	210	7.0
	8	10	1.3		20	92	4.6
	36	43	1.2				
Water supply reservoir (storm	91	96	1.1				
	32	44	1.4	Percent increase		93	

TABLE 3. *Verification of blue colonies from the two-layer agar*

Source	No. of colonies subcultured	Colonies as verified fecal coliforms	Percent verification
Raw sewage	538	477	88.7
Chlorinated effluent	70	69	98.6
River water	145	132	91.0
Marine water	80	79	98.8
Reservoir	180	173	96.1

be dependent on the kind(s) of bile salts used in the commercial formulation of the m-FC medium. Studies have been initiated to substantiate this observation and to ascertain the particular bile salts involved to standardize the medium formulation and minimize colonial variation.

The results indicate that the proposed two-layer agar membrane filter procedure allows for repair and subsequent reproduction of those fecal coliforms which have been debilitated by exposure to chlorine, industrial waste, or marine waters. Use of the two-layer medium concept automatically shifts the culture contact from an enrichment growth substrate to the essential differential medium phase. Further automation of the procedure could be realized by use of a temperature-programmed incubator that makes the change from 35 to 44.5 C after the initial 2-h incubation period. Following the 2-h incubation at 35 C the scheduled temperature increase to 44.5 C must be accomplished

within 45 min because of the critical requirement to base the fecal coliform concept on lactose fermentation at 44.5 C. Finally, the decision to use the slightly more involved two-layered medium procedure in preference to the direct m-FC method should be based on a demonstration of increased verified recovery of fecal coliforms in the samples routinely examined. However, with a major interest in the fecal coliform test being related to the bacterial quality assessment of effluents under the National Pollution Discharge Elimination System (a national permit program to control discharge of pollutants into the Nation's waters), the proposed technique could prove valuable.

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