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Evaluating the Membrane Fecal Coliform Test by Using *Escherichia coli* as the Indicator Organism

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The fecal coliform membrane filter method (MFC) currently used in water pollution analysis was evaluated by using two strains of *Escherichia coli*, a known fecal coliform, as the indicator organism. A large relative error in the results obtained with this method was found to be dependent upon the brand of membrane filter employed, the medium, and the temperature of incubation. MFC densities varied between 10 and 60% of the densities determined by means of total bacteria counts and total coliform counts performed at 35 C. Due to the large relative error encountered, the MFC method cannot be recommended as an analytical tool for the laboratory enumeration of *E. coli*. The results do show that the MFC method can be used at 35 C for enumeration of *E. coli* and for differential counts of *E. coli* and *Enterobacter aerogenes*.

The demonstration of the presence of coliform organisms in a water sample has been regarded as evidence of fecal pollution and has served for many years as a basis for water-quality criteria. Due to the lack of appropriate differential techniques that could quickly and specifically enumerate only cells of *Escherichia coli*, methods have generally tended to include other closely related species of *E. coli*-like or coliform organisms. Even though selective media were developed over the years that could distinquish the fecal indicator organism *E. coli* from *Enterobacter aerogenes* in the laboratory, they were less than adequate when employed in the field. For this reason these organisms are still combined into the coliform group that is defined as "all aerobic and facultative anaerobic, gram-negative, non-spore forming, rod-shaped bacteria that ferment lactose with gas formation within 48 hours at 37 C" (1).

Since coliforms are only indicative of fecal pollution and in some cases have not withstood rigid legal examination, it was deemed advisable to identify the organisms as *E. coli* (3). A major advance in this direction was made by Geldreich et al. (2) when they developed the fecal coliform membrane filter procedure (MFC). The Geldreich group did not claim this test was specific for *E. coli*, but rather that it detects the "fecal-coliform organism group." One of the main features of this method is its rather high and exacting incubation temperature requirements (44.5 ± 0.2 C). The MFC method was incorporated into the 13th edition of *Standard Methods for the Examination of Water and Wastewater* as an approved analytical procedure for water analysis (1).

Since *E. coli* is the most predominant member of the fecal coliform group of organisms, we were interested in investigating the possible use of this quantitative analytical tool for enumerating *E. coli* cells in other fields of microbiology (e.g., genetics, food and clinical microbiology, teaching, etc.). To be valid in these areas the accuracy must be better than 95%. The term "accuracy" is used here in its statistical sense, meaning that percentage of the actual number present that can be detected by the analytical method. The Geldreich group (2) studied the "productivity" and "selectivity" of the procedure. The productivity study was essentially an accuracy determination since they compared counts at 35 C on tryptose-glucose-extract (TGE) agar plates and at 44.5 C by using the MFC procedure. Their productivity values ranged from 75.7 to 100%, with an average 88% recovery.

Presswood and Brown (4) recently reported that the results they obtained by using Millipore filters (HAWG 047SO) were always lower than when they used Gelman filters (GN-6). The latter filter enumerated an average 2.3 times the number of cells in a given suspension than did the Millipore membrane. They also stated that colony counts on the Gelman filters agreed with control plate counts.

The Geldreich group did not report the exact procedures employed in isolating the fecal coli-

forms they employed for studies concerned with productivity of the MFC method (2). The implication, however, was that if the cultures were not isolated by the MFC procedure, they were at least selected by that procedure before being examined. Presswood and Brown (4) stated that their cultures were isolated by the MFC method. Such a procedure is not wise nor even necessary. Selection for mutant strains would result in populations of cells giving more favorable results on the same isolation medium.

It is also very important that the controls that are being used to determine the absolute number of viable cells in the suspension be a standard, accurate method. The Geldreich group assured this by employing standard plate counts (TGE agar) at 35 C. Presswood and Brown, however, used MFC medium pour plates at 35 C.

This study differs from those reported previously in the following respects. The test organism, a "fecal coliform" according to the definition, was grown at 35 C. The true value for the absolute number of viable cells in a given suspension was obtained by using plate count medium at 35 C. In this way we hoped to remove any experimental bias in determining the accuracy of the MFC procedure. Our results also differ from those reported previously.

MATERIALS AND METHODS

Cultures. The indicator organisms employed in this study included *E. coli* (ATCC 11775), *E. aerogenes* (ATCC 13048), and an isolate from lake water that was identified as a fecal coliform and designated as R-273.

Culture *E. coli* (11775) is the neotype strain of *E. coli*. Our laboratory stock was examined for purity by plating on Levine eosin methylene blue (EMB) (Difco), by acid and gas formation in lactose broth, and by performing the indole–methyl red–Voges-Proskauer–citrate utilization (IMViC) test. The culture exhibited a definite green, metallic sheen on EMB and produced acid and gas in lactose broth. It was classified as *E. coli* variety 1 (IMViC ++−−). It was also classified as a fecal coliform after confirmation by acid and gas production at 44.5 C in lactose broth.

Culture *E. aerogenes* (13048) is the suggested neotype strain of *E. aerogenes*. It was classified as *E. aerogenes*, variety 1 (IMViC −−++). Purity of the culture was determined by streaking on EMB agar plates.

Culture (R-273) was isolated from lake water by membrane filtration and incubation at 44.5 C on MFC medium. A typical blue colony was isolated and shown to produce acid and gas in lactose broth at 35 and 44.5 C. It was classified as fecal coliform variety 1 (IMViC ++−−).

Culture media. Cultures were prepared by growing the cells overnight in nutrient broth. The *E. coli* cultures and cultures of R-273 were incubated at 35 C. The *E. aerogenes* cultures were incubated at room temperature.

For enumeration of the total number of viable cells present, m-plate count broth (Difco) and M-coliform broth (BBL) were employed. Counts were made by using the membrane filter technique. Incubations were at 35 C. For comparing the accuracy of the MFC method, cell densities were determined on m-plate count broth (Difco) and MFC broth base (Difco and BBL) containing 0.01% rosolic acid. Incubations were at 44.5 C.

Membrane filters. Two different brands of filters were used in this study. The Millipore filters were type HAWG 047SO (Millipore Corp., Bedford, Mass.). These filters were sterilized by the manufacturer by using ethylene oxide. Some were re-sterilized by autoclaving before they were used, and no differences due to autoclaving were noted. The Gelman filters were type GA-6 (Gelman Instrument Co., Ann Arbor, Mich.) and were sterilized by autoclaving in our laboratory.

Procedure. A 10^{-6} dilution of a sample of an overnight culture was made in sterile phosphate buffer (3.4×10^{-4} M KH_2PO_4, pH 7.2). Quantities of 10, 25, and 100 ml were filtered, using a Millipore filtering apparatus, to obtain a total of 20 to 60 colonies on each membrane. In some experiments, where fewer cells were available, a 10^{-5}, 10^{-7}, or 10^{-8} dilution was employed. Replicate filters were made of each count. The number of replicates varied between two and six, depending upon the experiment. Reported values are the average of all replicate plates employing the same filter type, temperature, and medium, multiplied by a factor that would give the result as the number of cells per 100 ml of buffer. This is in keeping with current practice in water pollution analysis (1). Random placing of the filters on the different media and random selection for incubation at different temperatures was used to remove bias.

The membranes were incubated in a Psychro-Therm incubator (New Brunswick Scientific Co., New Brunswick, N.J.) at 35.0 ± 0.5 C or in "Whirl-Pak" bags in a circulating water bath, constructed by the author, at 44.51 ± 0.02. The water bath temperature was monitored by a Hewlett-Packard 2801-A Quartz thermometer. Standard methods were followed at all times.

RESULTS

Since the accuracy of an analytical procedure is defined as the agreement between the quantity measured by the test and the quantity actually present (expressed as the relative error, in percent), it is extremely important that the quantity present (i.e., the exact number of bacterial cells) be known. In this study we have made the assumption that the results obtained with either the plate count broth or the coliform broth at 35 C on Millipore membranes represent the actual number of viable cells in the buffer solution. The results shown in Table 1

TABLE 1. *Comparison of E. coli cell counts on plate count and total coliform media and on different brands of membrane filters at 35 C*

Expt no.	Filter type	No. of bacteria per 100 ml	
		Plate count broth	Total coliform broth
1	Millipore		50
	Gelman		48
2	Millipore		24
	Gelman		20
3	Millipore		79
	Gelman		73
4	Millipore	121	121
5	Millipore	124	124
6	Millipore	51	54
7	Millipore	162	167
8	Millipore	90	93
9	Millipore	83	99
10	Millipore	97	83
11	Millipore	140	130
12	Millipore	67	66
13	Millipore	98	88
14	Millipore	110	118

support the conclusion that little variation is found between these two media when using Millipore membranes or between the two different brands of membranes when grown at this temperature. The first three experiments in Table 1 show the results obtained when comparing Gelman and Millipore filters at 35 C using coliform broth. Little variation is found between the membranes under these conditions. In experiments 4 through 14, only Millipore membranes were employed. Although some trials show variation between the two different media, neither medium consistantly showed higher counts. The differences are therefore assumed to result from random error. In these trials the differences seen are not statistically significant.

Table 2 shows the results obtained in experiments designed to determine differences in the counts obtained with the standard total coliform analysis and the new fecal coliform analysis. Millipore filters were employed. Total bacterial counts that were incubated at both 35 and at 44.5 C served as controls. If the filter membrane or the incubation temperature alone had any effect, the total bacterial count at 44.5 C would be expected to be less than the count at 35 C. Table 2 shows that there was an effect, and a reduced count was obtained. A more dramatic and consistant reduction in viable count was seen when the MFC medium was employed at 44.5 C. Total counts at 44.5 C approximated 25% of the actual number of viable cells in the suspension. Fecal coliform counts, on the other hand, were approximately 10% of the actual number. In several experiments fecal coliform counts as high as 25% of the actual number were obtained. In one of these, experiment 8, Millipore field monitors (Millipore Corp., Bedford, Mass.) were used and 24.8% of the cells were detected. In the other, experiment 9, culture R-273 was employed. This culture consistantly gave higher counts at 44.5 C than did *E. coli* 11775. In this case, 21% of the cells formed colonies.

A comparison of Gelman and Millipore filters at 35 and 44.5 C is shown in Table 3. Although both filters show a large relative error at 44.5 C, the difference in performance between the two

TABLE 2. *Comparison of E. coli densities determined by the total coliform method at 35 C and the fecal coliform method at 44.5 C*

Expt no.	No. of *E. coli* cells per 100 ml			
	35 C		44.5 C	
	Plate count broth	Total coliform broth	Plate count broth	MFC broth
1	78	88	20	7
2	98	88	26	6
3	67	66	11	6
4		83		5
5	97	83		7
6	51	54		6
7	162	167		6
8	196	192		48[a]
9	74	81		17[b]

[a] Millipore field monitors used for filtration.
[b] Culture R273 was used for this experiment.

TABLE 3. *Relative error in the fecal coliform method as a function of the brand of membrane filter employed*

Expt no.	Filter brand	No. of *E. coli* cells per 100 ml		Relative error[a] (%)
		Total coliform broth (35 C)	MFC broth (44.5 C)	
1	Millipore	50	6	88%
	Gelman	48	26	46
2	Millipore	46	5	89
	Gelman	48	21	56
3	Millipore	79	6	92
	Gelman	73	50	32

[a] Relative error = count 35 C − count 44.5 C/count 35 C.

brands is quite dramatic. The relative error when using Millipore filters was close to 90%. Only 10% of the cells were actually being counted. The relative error with Gelman filters was less, but more variable. Counts between 44 and 68% of the actual number were obtained when using the Gelman membrane.

Since several of the earlier investigators examining the MFC method employed organisms obtained by isolation at 44.5 C on the fecal coliform medium which could lead to the isolation of mutants better able to grow under the conditions of the test, we have investigated the effects of growing the *E. coli* culture at 44.5 C before counting. This does not rule out the effects of the filter or the medium as selective agents, but it does show any effects that temperature alone might have. Table 4 shows the results of these high-temperature growth experiments. A culture (tube no. 1) was inoculated and incubated at 44.5 C for 24 h. This tube was counted and used to inoculate tube no. 2, and both tubes were reincubated. After 48 h, tubes no. 1 and 2 were counted and tube no. 3 was inoculated from tube no. 2. All tubes were reincubated for 24 h, and this process was repeated again with tube no. 4. The times given represent the time each culture tube had been at 44.5 C.

These data show a dramatic increase in the percentage of cells capable of growth at the higher temperature. Growth of the culture at 44.5 C for 120 h, while resulting in a decrease in the total number of viable cells, increased the MFC-positive cells from 13 to 60%. Serial culture of the organism at this temperature did not result in as large an increase. Tube no. 4, grown for 24 h, contained 38% MFC-positive cells. This was an increase over the 13% occurring in the original culture.

To determine what effect temperature itself contributed to the inaccuracy of the MFC procedure, experiments were performed in which all of the incubations were at 35 C. Table 5 shows the results of this study. Cultures of *E. aerogenes* were also employed in this study to see if the medium could differentiate between these two organisms at the lower temperature. In all cases the *E. aerogenes* colonies were cream to light green in color. In mixtures of the two organisms, good differential counts were obtained. There was no apparent difference between counts of *E. coli* made on total count broth at 35 C and fecal coliform broth at 35 C.

TABLE 4. *Recovery data for E. coli grown at 44.5 C*

Tube no.[a]	Incubation time (h) at 44.5 C	No. of *E. coli* cells per 100 ml[b]		$\frac{FC}{TC} \times 100$[c]
		Total coliform (35 C)	MFC (44.5 C)	(%)
1	24	1,180	158	13
	72	300	30	10
	96	20	9	45
	120	30	18	60
2	48	920	85	9
	72	240	62	27
	96	110	22	20
3	24	1,780	490	28
	48	510	113	22
4	24	1,440	540	38

[a] Each tube is a serial inoculation of the previous tube.

[b] Counts are given as cells per 100 ml of a 10^7 dilution.

[c] Fecal coliforms/total coliforms.

DISCUSSION

At first glance it would appear that the MFC procedure offers a new laboratory technique in areas other than water pollution microbiology. The relatively high selectivity of the procedure demonstrated in the earlier studies by Geldreich et al. (2) suggests its use in those quantitative studies, using *E. coli*, in which manipulation procedures offer chances for excessive contamination. To serve in such a capacity it would be necessary that the procedure be quantitative. Our initial investigations showed a large discrepancy between the number of *E. coli* cells present in a particular suspension and the number that could be detected by

TABLE 5. *Evaluation of the differentiation of E. coli and E. aerogenes by the MFC method at 35 C*

Expt no.	Culture	No. of cells per 100 ml	
		Plate count broth (35 C)	MFC broth (35 C)
1	*E. coli*	58	59
2	*E. coli*	51	48
3	*E. aerogenes*	59	58[a]
4	*E. coli*		73
	E. aerogenes		154[a]
	Mixture (1:1)		230[a]
5	*E. coli*		156
	E. aerogenes		253[a]
	Mixture (1:1)		389[a]

[a] *E. aerogenes* colonies were cream to light green in color.

means of the MFC procedure. Because this difference was variable it was not possible to estimate the true number of cells present from the results of plate counts using the MFC method. Two strains of *E. coli* were used.

After our studies began, reports appeared in the literature that showed deficiencies in the MFC test (4; S. A. Shahidi and M. H. Backer, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 9, 1973). Presswood and Brown (4) reported that variation in cell densities were based on the brand of filter employed. They compared the Gelman and Millipore brands. We extended our own studies to compare these same two brands of filters. One important difference in the approach used in that study and this report is in the source of the fecal coliform organisms. It is doubtful whether our results can be directly compared with theirs because their use of the MFC procedure to isolate the indicator organisms would probably have selected for mutants better able to cope with the restrictive assay conditions. It is obvious from their study, however, that an interaction does occur with the Millipore filter, especially at 44.5 C.

When we compared these two filters at 44.5 C (Table 3), we also obtained differences in the cell counts on the different brands of filters. Neither brand of filter, however, gave adequate results. Unlike Presswood and Brown, we have not been able to find any differences in the results obtained with the two brands of filters when the incubations are performed at 35 C. Differences in the type of Gelman membrane we used (GA-6) and their membrane (GN-6) could be significant. We recommend that investigators request the manufacturer to check and guarantee each order for the procedures in which he employs these filters. The method employed for this evaluation should be stated clearly.

It is interesting to speculate as to the reason that all of the cells in the suspension do not propagate under the conditions employed in the MFC procedure. If one considers the higher temperature, the membrane filter effects, the ingredients of the medium, and the interaction between some or all of these factors, there are a wealth of possibilities. We have shown that the medium works at 35 C and that the differences between the two brands of filters is not seen at the lower temperature. Although this would seem to point to the effect of temperature, it is not apparent whether the effect of temperature is directed toward the cell, the membrane, or the medium. In the case of the Millipore membrane, it is apparent that a membrane-associated inhibitory effect is involved. This could also be the case with the Gelman filter, but to a lesser degree.

The results of our study on high-temperature mutant selection bear out our concern about comparing our results with previous studies. As Table 4 demonstrates, the number of cells that can grow and form colonies in the MFC test, as a percentage of the total viable population, can increase with prior incubation at 44.5 C. Tube no. 1 represents a culture that was maintained at 44.5 C for 120 h. During that time the number of viable cells was decreasing. The ratio between those recovered by the two different methods was increasing to a maximum of 60%. Tube no. 2 represents a culture inoculated from tube no. 1 at 24 h. Tube no. 3 represents a culture inoculated from tube no. 2 after it had incubated 48 h, etc. The cells in tube no. 4 represent a culture that has been selected for high-temperature growth by three serial cultivations at 44.5 C over a period of 72 h. After only 24 h the ratio of cells capable of growth at 44.5 C to total cells was 38%. These results demonstrate that prior incubation at 44.5 C does alter the percentage of cells that can be recovered by the MFC procedure. We have never been able to select for a culture in which all of the cells will grow at 44.5 C on either the total count or the fecal coliform medium. These experiments always employed the Millipore brand of filters, but other experiments (Table 3) suggest that even better results would have been obtained had we used the Gelman filter.

The MFC procedure works quite well in differentiating the present strain of *E. coli* from *E. aerogenes* when performed at the lower temperature (Table 5). We do not know what problems would be encountered with other strains at this temperature, or from other species if the lower temperature is used in the field. However, for laboratory differentiation and enumeration of these two organisms, the 35 C incubation would appear to be advantageous. In a more recent report, the Geldreich group (D. J. Van Donsel, R. M. Twedt, and E. E. Geldreich, Bacteriol. Proc., p. 25, 1969) advocated an incubation temperature at 41.5 C for a new 7-h fecal coliform method. Since the medium is different from that employed in their MFC procedure, no direct comparison can be made with our results. They showed that the same number of colonies were obtained with both the 7-h method and the MFC method.

In conclusion, we do not doubt that the MFC procedure can provide valuable data in the analysis of polluted water in the field, but we do find that its lack of quantitative accuracy makes it less than desirable as an analytical tool

in the laboratory. We have not found any cautions to investigators in any of the published papers, instructions, or methods manuals that would alert individuals to this fact. We also feel that our studies and those of earlier investigators show a need for having membrane filters "procedure-tested" and certified by the manufacturer.

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