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Improved Membrane Filtration Media for Enumeration of Total Coliforms and *Escherichia coli* from Sewage and Surface Waters†

TIMOTHY A. FREIER AND PAUL A. HARTMAN*
Department of Microbiology, Iowa State University, Ames, Iowa 50011

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Two media were developed that allowed both a total coliform count and an *Escherichia coli* count to be determined on the same medium after 24 h of incubation at 35°C. The new media were tested along with two standard media on 10 surface water and 7 sewage samples. The experimental media yielded equivalent or higher counts relative to the standard media and recovered more specifically the desired indicator groups as determined by colony identification.

Current membrane filtration methods have been criticized for their many shortcomings (for a review, see reference 15). Several investigators have suggested that *Escherichia coli* be used as an indicator because it is specific for fecal pollution and provides a more accurate indicator-to-pathogen ratio than total or fecal coliform enumeration methods (4, 7, 9).

Increased attention is being given to the recovery of injured indicator bacteria (3, 5, 6, 19, 20, 22, 23). One of the major factors causing reduced recoveries of injured bacteria is the selective agent(s) that is used. Monensin is an ionophore that selectively inhibits all gram-positive bacteria while it allows growth of injured gram-negative bacteria (25). Tergitol 7, a surfactant, has a similar effect (17, 19).

Recently, a very sensitive and specific method for the detection of β-glucuronidase production by *E. coli* was developed (10, 15, 25). Over 95% of *E. coli* hydrolyze a nonfluorescent substrate, 4-methylumbelliferyl-β-D-glucuronide (MUG), to produce 4-methylumbelliferone, which fluoresces under long-wave UV light. Approximately 50% of *Shigella* spp., some *Salmonella* spp., and a few strains of *Yersinia enterocolitica* are the only other members of the families *Enterobacteriaceae* and *Vibrionaceae* that produce this enzyme (12, 14, 18, 21, 25, 29).

The purpose of this study was to develop an improved membrane filtration method by using media that contain monensin, Tergitol 7, and MUG. The goal was the enumeration of total coliforms and *E. coli* on a single medium incubated at 35°C with improved recovery of injured cells and improved selective specificity when compared with standard membrane filtration media and methods.

MATERIALS AND METHODS

Media. MUG was obtained from Hach Co. (Loveland, Colo.). Monensin (90 to 95% pure) was obtained from Sigma. All media were prepared with 1.5% agar, sterilized, tempered to 47°C, and poured in approximately 3-ml volumes into petri plates (diameter, 47 mm).

Peptone-Tergitol-glucuronide (PTG) agar was prepared as described previously (5). Peptone glucuronide (PG) agar was prepared exactly as PTG agar, except that the Tergitol 7 (J. T. Baker Chemical Co., Phillipsburg, N.J.) was omitted. PG agar was poured into standard petri plates (diameter, 100 mm) for the isolation and purification of colonies. Enriched lauryl sulfate-analine blue (ELSAB) agar was prepared as described by Wright (31). Tergitol 7 agar (m-T7) was prepared initially as described previously (19); later, it was obtained from Difco Laboratories (Detroit, Mich.). m-FC broth base and m-Endo broth MF were obtained from Difco and prepared with 1.5% agar. m-FC agar was used without rosolic acid. The formulation of lactose-monensin-MUG (m-LMM) agar, which was autoclaved for 15 min at 121°C (final pH, 7.2), was as follows: Tryptone (Difco), 5.0 g; yeast extract, 2.5 g; lactose, 10.0 g; bromocresol purple, 80.0 mg; K_2HPO_4, 3.3 g; KH_2PO_4, 1.0 g; MUG, 100.0 mg; agar, 15.0 g; monensin, 38.0 mg (in 10 ml of 95% ethanol); distilled water, 1,000.0 ml. Tergitol-monensin-MUG (m-TMM) agar was prepared exactly as m-LMM, except that 0.25 ml of Tergitol 7 per liter was added before sterilization. m-TEC agar was prepared as described previously (8). Starch ampicillin (SA) agar (24) was used for the presumptive identification of *Aeromonas hydrophila*. A modified standard plate count medium (m-SPC) was made by the method of Taylor and Geldreich (28). All dilutions were made in 0.1% peptone water.

Sample collection. Samples were collected in sterile 200-ml glass screw-cap bottles. Surface water samples were collected from lakes, streams, and rivers in central Iowa. Sewage samples were collected from various treatment stages at the Ames, Iowa, and the Nevada, Iowa, wastewater treatment plants, both of which process residential and light industrial wastes, including a moderate quantity of surface water runoff. The sewage effluents were not chlorinated. An additional sewage sample was collected from a lagoon serving a small residential community. The samples were tested to determine the approximate coliform and *E. coli* levels and were stored overnight at 4°C. The next day (within 28 h of collection), the samples were subjected to extensive comparative studies.

Media comparison. On the basis of preliminary experiments reported below (MUG incorporation), PTG, m-LMM, and m-TMM agars were selected for comparison with m-Endo and m-FC agars in more extensive tests. A total of 10 surface water samples and 7 sewage samples were collected over a 7-month period. Fifteen filters were prepared from each of several appropriate dilutions. The filters were placed on triplicate plates of the three trial and two standard media. The plates were incubated at 35°C, except for m-FC plates, which were incubated in a 44.5°C circulating water bath for 18 to 24 h. Typical lactose-positive reactions were counted as dark blue colonies on m-FC agar, green colonies with a

* Corresponding author.
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TABLE 1. Geometric mean coliform counts obtained on three media before and after correction for *A. hydrophila*

Sample	No. of coliforms/100 ml after growth on:		
	m-Endo	m-LMM	m-TMM
Sewage[a]	6.6×10^6	4.3×10^6	5.5×10^6
Sewage[b]	5.2×10^6	4.1×10^6	5.2×10^6
Surface water[a]	9.3×10^3	9.1×10^3	9.3×10^3
Surface water[b]	5.6×10^3	8.5×10^3	9.1×10^{3c}

[a] Uncorrected counts.
[b] Corrected counts after subtraction of the proportionate number of lactose-positive *A. hydrophila* as determined for each sample.
[c] Significant increase over m-Endo count ($P < 0.1$).

metallic sheen on m-Endo agar, and yellow colonies on m-LMM and m-TMM agars. Fluorescent colonies were detected by placing the plates under a long-wave UV light. To enhance fluorescence, an absorbant filter pad was placed in the cover of a dish, 3 to 5 drops of undiluted ammonium hydroxide (30% NH_3) were placed on the pad, and the plate to be counted was placed over it for 10 to 15 s.

Colony identification. Isolated colonies were picked from the most appropriate dilution of each medium type. For each sample, five lactose-positive, MUG-positive colonies (presumptive *E. coli*); five lactose-positive, MUG-negative colonies (presumptive coliforms); and five lactose-negative, MUG-negative colonies (presumptive noncoliforms) were chosen at random from m-LMM and m-TMM agars. From m-Endo and m-FC agars, five lactose-positive and five lactose-negative colonies were chosen at random from each medium type. Five MUG-positive and five MUG-negative colonies were picked at random from PTG agar plates.

Colonies picked from the various media were struck for isolation on plates of PG agar. The plates were incubated for 18 to 24 h at 35°C and then were examined under white and UV light. Pure cultures were tested for the production of cytochrome oxidase. Oxidase-positive cultures were struck on plates of SA agar, and the plates were incubated for 24 h at 30°C and were then flooded with Lugol iodine solution. Colonies that were capable of growth on ampicillin and that exhibited zones of clearing (amylase positive) were recorded as *A. hydrophila*. Oxidase-positive cultures that were negative for growth or amylase production on plates of SA agar were inoculated for identification in Rapid NFT strips (Analytab Products, Plainview, N.Y.). Oxidase-negative cultures were inoculated in API 20E (Analytab) strips.

Chlorination study. To determine recoveries of chlorine-injured cells on various media, the method of Camper and McFeters (3) was used, except *E. coli* B was injured at a chlorine concentration of 2.6 mg/liter for 6 min, and the diluent used was 0.1% peptone water. Dilutions of control and injured cells were dispersed in 100-ml peptone blanks; drawn through membrane filters; and placed on m-SPC, m-FC, m-Endo, PTG, m-T7, m-LMM, and m-TMM agars. All plates were incubated at 35°C for 24 h, except that m-FC agar plates were incubated at 44.5°C in a circulating water bath. This experiment was repeated three times.

Statistical analysis. The media comparison data and the chlorination study data were analyzed by analysis of variance. Natural logarithmic transformations of the observed counts were analyzed in a randomized complete block design (27). Differences in the mean recoveries on the various media were compared by using the Student *t* test.

RESULTS

MUG incorporation. The optimal concentration of MUG for incorporation into m-LMM and m-TMM agar was 100 μg/ml. Fluorescent reactions on m-TEC, ELSAB, m-T7, m-Endo, and m-FC agars were difficult to observe, even at a MUG concentration of 200 μg/ml. MUG-positive colonies on PTG were bright and easy to detect at a MUG concentration of 50 μg/ml, as reported by Damaré et al. (5). During the preliminary experiments, several colonies with various morphologies were picked from the experimental media to be Gram stained. Gram-positive colonies were never isolated from ELSAB, m-TEC, m-T7, m-LMM, or m-TMM agars. A small number of pinpoint colonies on PTG agar were gram positive; however, these usually were not visible until incubation periods exceeded 24 h.

ELSAB and m-TEC agars were eliminated from further studies because of low recoveries and the poor visualization of the MUG reaction on these media (data not shown). Recoveries on m-T7 agar containing MUG were excellent, but the MUG reactions were poor.

Recovery comparisons. Coliform recoveries on m-Endo, m-LMM, and m-TMM agars are shown in Table 1. PTG agar was differential only for *E. coli*, not for total coliforms; therefore, PTG agar counts were not included in the total coliform results. Because of the large numbers of *A. hydrophila* recovered on m-Endo agar, the total coliform counts were corrected to show the number of true coliforms that were recovered (Table 1). m-TMM coliform counts were significantly higher than m-Endo counts for surface waters.

The recoveries of fecal coliforms on m-FC agar and *E. coli* on PTG, m-LMM, and m-TMM agars are shown in Table 2. PTG and m-LMM agars yielded significantly greater recoveries of *E. coli* than total fecal coliforms on m-FC agar for surface water samples.

Species distribution. Species distributions of lactose-positive bacteria isolated from each medium differed as shown in Tables 3 and 4. On m-LMM and m-TMM agars a higher percentage of the genera normally classified as coliforms was recovered.

Percentages of *A. hydrophila* isolated on the five media are

TABLE 2. Geometric mean counts of fecal coliforms and *E. coli* on four media

Sample	m-FC (no. of Lac^+/100 ml)[a]	No. of MUG^+/100 ml after growth on[b]:		
		PTG	m-LMM	m-TMM
Sewage	3.5×10^5	5.1×10^{5c}	3.5×10^5	3.8×10^5
Surface water	9.7×10^2	1.7×10^{3c}	2.1×10^{3d}	1.1×10^3

[a] Characteristic blue colonies were counted as fecal coliforms.
[b] Characteristic blue halos under long-wave UV light were counted as *E. coli*.
[c] Significant increase over m-FC count ($P < 0.1$).
[d] Significant increase over m-FC count ($P < 0.05$).

TABLE 3. Lactose-fermenting organisms isolated from samples of surface water on four media

Organism	No. of isolates (%) after growth on:			
	m-Endo	m-FC	m-LMM	m-TMM
Aeromonas hydrophila	14 (32.5)	0 (0)	2 (2.3)	2 (2.2)
Citrobacter amalonaticus[a]	1 (2.3)	0 (0)	0 (0)	1 (1.1)
Citrobacter freundii[a]	6 (14.0)	0 (0)	8 (9.1)	6 (6.7)
Enterobacter aerogenes	0 (0)	0 (0)	0 (0)	1 (1.1)
Enterobacter agglomerans	0 (0)	1 (2.0)	3 (3.4)	12 (13.5)
Enterobacter cloacae[a]	2 (4.7)	0 (0)	9 (10.2)	5 (5.6)
Enterobacter sp.	0 (0)	0 (0)	1 (1.1)	0 (0)
Escherichia coli[a]	11 (25.6)	47 (92.2)	54 (61.4)	48 (53.9)
Klebsiella oxytoca[a]	4 (9.3)	0 (0)	6 (6.8)	5 (5.6)
Klebsiella pneumoniae[a]	3 (7.0)	3 (5.9)	3 (3.4)	7 (7.9)
Providentia stuartii	1 (2.3)	0 (0)	0 (0)	0 (0)
Serratia fonticola	1 (2.3)	0 (0)	1 (1.1)	0 (0)
Serratia liquefaciens	0 (0)	0 (0)	1 (1.1)	1 (1.1)
Serratia rubidaea	0 (0)	0 (0)	0 (0)	1 (1.1)

[a] Coliform (as defined by Gavini et al. [11]).

shown in Table 5. A slightly higher percentage of *A. hydrophila* was recovered from sewage samples than from surface water samples on m-LMM and m-TMM agars. However, percentages of *A. hydrophila* were much lower on m-LMM and m-TMM agars than on m-Endo agar or PTG agar. No *A. hydrophila* were recovered on m-FC agar.

The use of the MUG reaction to identify *E. coli* was specific (data not shown). Only one MUG-positive non-*E. coli* was isolated during this study. It was typed as *Shigella sonnei* and was isolated from a sewage sludge digestor sample. Of 224 *E. coli* isolated and identified from surface waters, 7.7% were MUG negative and 9.8% were lactose negative. Sewage samples yielded 11.2% MUG-negative and 12.6% lactose-negative *E. coli* of the 129 isolates identified from this source.

Chlorine injury study. The abilities of seven different media to recover chlorine-injured cells are shown in Table 6. On m-FC agar a significantly lower number of cells was recovered compared with the other six media even before chlorination ($P < 0.05$). The highest counts were obtained on m-SPC agar, which is a nonselective medium. As expected, on m-Endo and m-FC agars small percentages of injured cells were recovered (40 and 24%, respectively). On PTG, m-T7, m-LMM, and m-TMM agars, significantly greater numbers of injured cells were recovered ($P < 0.05$).

TABLE 4. Lactose-fermenting organisms isolated from samples of sewage on four media

Organism	No. (%) of isolates after growth on:			
	m-Endo	m-FC	m-LMM	m-TMM
Aeromonas hydrophila	6 (18.3)	0 (0)	2 (3.3)	3 (5.0)
Centers for Disease Control enteric group 17	1 (3.0)	0 (0)	0 (0)	1 (1.7)
Citrobacter sp.	1 (3.0)	0 (0)	0 (0)	0 (0)
Citrobacter freundii[a]	1 (3.0)	1 (3.2)	8 (13.1)	7 (11.7)
Enterobacter aerogenes	1 (3.0)	0 (0)	0 (0)	1 (1.7)
Enterobacter agglomerans	1 (3.0)	0 (0)	2 (3.3)	0 (0)
Enterobacter cloacae[a]	4 (12.1)	0 (0)	6 (9.8)	3 (5.0)
Escherichia coli[a]	5 (15.2)	24 (77.4)	36 (59.0)	32 (53.3)
Kluyvera sp.	0 (0)	0 (0)	1 (1.6)	1 (1.7)
Klebsiella oxytoca[a]	8 (24.3)	0 (0)	3 (5.0)	2 (3.3)
Klebsiella ozaenae	1 (3.0)	0 (0)	1 (1.6)	0 (0)
Klebsiella pneumoniae[a]	3 (9.1)	5 (16.2)	2 (3.3)	9 (15.0)
Serratia liquefaciens	0 (0)	0 (0)	0 (0)	1 (1.7)
Serratia oderifera	1 (3.0)	0 (0)	0 (0)	0 (0)
Shigella sonnei	0 (0)	1 (3.2)	0 (0)	0 (0)

[a] Coliform (as defined by Gavini et al. [11]).

DISCUSSION

Increased recoveries of total coliforms were obtained on m-TMM agar when compared with m-Endo agar when surface water samples were examined. Increased recoveries of fecal coliforms were obtained from surface waters on PTG and m-LMM agars when compared with m-FC agar and from sewage on PTG agar. The surface water samples probably contained a higher number of injured cells than the sewage samples.

A significantly different spectrum of bacterial species was recovered on the monensin-containing media (m-LMM and m-TMM) in comparison with the Tergitol 7-containing medium (PTG agar; data not shown) and the standard media. The most important difference was the recovery of *A. hydrophila*; large proportions of coliform-positive colonies on m-Endo agar and total colonies on PTG agar were *A. hydrophila*. *Aeromonas* spp. usually are excluded from the coliform group (11) because they are not normally found in the feces of healthy humans and would not, therefore, indicate fecal pollution (1). *A. hydrophila* can also multiply in the environment (2). A method to enumerate specifically *Aeromonas* spp. in the presence of closely related bacteria has been developed (26). Because m-LMM and m-TMM agars recovered low numbers of aeromonads, background interference was reduced, and the counts that were obtained reflected more accurately the sanitary history of the water sample. A greater diversity of sample types and locations should be examined to determine if this increased specificity is universal and consistent.

TABLE 5. Numbers and percentages of lactose-positive and -negative *A. hydrophila* isolated from surface waters and sewage from five different media

Medium	No. (%) of *A. hydrophila* from:	
	Surface waters	Sewage
m-Endo	45 (52.3)	34 (50.8)
m-FC	0 (0)	0 (0)
PTG	16 (18.2)	17 (28.8)
m-LMM	5 (3.6)	9 (9.5)
m-TMM	5 (3.6)	10 (10.6)

TABLE 6. Recoveries of chlorine-injured *E. coli* B on seven media

Medium	Count/100 ml:		
	Before chlorination[a]	After chlorination[a,b]	% Injured cells recovered[c]
m-SPC	7.1×10^{9}[d]	1.2×10^{7}[d,e]	100.0
m-Endo	6.8×10^{9}[d]	4.8×10^{6}	39.9
m-FC	4.1×10^{9}	2.9×10^{6}	24.3
PTG	6.0×10^{9}[d]	7.3×10^{6}[d]	60.8
m-T7	6.8×10^{9}[d]	9.0×10^{6}[d,e]	75.3
m-LMM	6.1×10^{9}[d]	7.6×10^{6}[d]	63.7
m-TMM	6.8×10^{9}[d]	7.6×10^{6}[d,f]	63.3

[a] Arithmetic means of three runs.
[b] Treated with 2.6 mg of chlorine per liter for 6 min at 25°C.
[c] (Count on selective media/count on m-SPC) × 100.
[d] Very significant increase over m-FC count ($P < 0.05$).
[e] Very significant increase over m-Endo count ($P < 0.05$).
[f] Significant increase over m-Endo count ($P < 0.1$).

When *E. coli* are enumerated on PTG, m-LMM, or m-TMM agars, plates should be examined for fluorescent colonies, even if the total number of colonies is too numerous to count. If *E. coli* is present as a small fraction of the coliform population of a sample, separate dilutions must be prepared for the total coliform and *E. coli* counts. The membranes should be examined after 22 to 24 h of incubation. Care should be taken not to count fluorescent pseudomonads, which appear as small, green-glowing colonies under long-wave UV light. The use of NH_4OH aids in distinguishing between colonies of fluorescent pseudomonads and true MUG-positive colonies. All colonies with any yellow or green coloration under white light should be counted as coliforms on m-LMM and m-TMM agars. Noncoliforms will appear smaller and purple. It has been recommended that 0.1% peptone water rather than a diluent containing potassium or sodium be used in conjunction with monensin-containing media (25).

An important advantage of using one of the three new media tested in this study is that no resuscitation step is needed, thus reducing time and expense. The ability to enumerate *E. coli* (fecal coliforms) at 35°C, rather than at 44.5°C, increases recovery of injured cells without the use of a low-temperature resuscitation step (16).

The determination of *E. coli* directly provides a more accurate indication of fecal pollution than do determinations of fecal coliforms based on their ability to grow at high temperatures. High counts of thermotolerant *Klebsiella* spp. have been reported in waters that were not polluted by fecal material (4, 30).

The recovery of chlorine-injured cells on m-LMM and m-TMM agars was slightly below that on m-T7 agar, although the difference was not statistically significant for this set of experiments. Unfortunately, because of the formulation of m-T7 agar, lactose-fermenting colonies produced large quantities of acid, and the MUG reaction was often difficult and sometimes impossible to detect. The fluorescence of 4-methylumbelliferone is highly dependent on pH (13). More work needs to be done to determine the efficacy of the use of these new media in recovering indicator cells in chlorinated tap waters.

For enumeration of both total coliforms and *E. coli* on a single medium at 35°C, m-LMM and m-TMM agars should be used side by side on representative samples to determine which medium is best for a specific sample category. The use of these media eliminates the need for a critically controlled 44.5°C incubator and the need for two membranes and media for each sample (one for total coliforms and another for fecal coliforms) and also offers increased specificity.

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