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Evaluation of m-T7 Agar as a Fecal Coliform Medium

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m-T7 agar, designed to improve recoveries of injured total coliforms, was evaluated for its effectiveness as a fecal coliform medium. The time and temperature of preincubation were found to be crucial to the optimal recovery of fetal coliforms. Isolation rates for fecal coliforms on m-T7 agar from sewage effluents were the highest when plates were preincubated at 37°C for 8 h before transfer to 44.5°C for 12 h. The medium was found to produce consistently higher fecal coliform counts than all the other methods tested. Recoveries were 3.1 times greater than the standard m-FC method and 1.7 times greater than the two-layer enrichment, temperature acclimation procedure. Verification rates for fecal coliforms isolated on m-T7 agar averaged 89.0%, whereas verification rates for m-FC agar averaged only 82.8%. Both media isolated similar fecal coliform populations. The advantages of a single medium, highly effective for the isolation of both total and fecal coliforms, are discussed.

It is well recognized that coliforms in chlorinated sewage effluents may be stressed or injured and may not grow when selective media are used (2–4, 8). In 1973, Lin (8) observed that fecal coliform levels from chlorinated sewage effluents enumerated by the membrane filter (MF) technique with m-FC medium at 44.5°C were lower than fecal coliform recoveries by the elevated temperature, multiple-tube procedure. Braswell and Hoadley (3) reported that *Escherichia coli* cells injured during chlorination of secondary sewage failed to produce colonies on m-FC agar or to grow and produce gas in lactose broth.

Under nonrestrictive conditions, injured coliforms can regain the ability to grow on selective media through a resuscitation process (2, 4). As a result, various resuscitation techniques have been proposed (6, 9, 11–13). Rose et al. (12) reported an improved fecal coliform MF method that used m-FC agar with a lactose broth agar overlay. Plates were preincubated at 35°C for 2 h, at which time the temperature was increased to 44.5°C for 22 to 24 h. When tested with a variety of water samples, the two-layer method recovered almost twice the number of fecal coliforms compared with the standard MF technique. In 1976, Lin (9) proposed a similar resuscitation method with phenol red lactose broth and incubation at 35°C for 4 h, followed by plating on m-FC agar at 44.5°C for an additional 18 h. This two-step MF technique produced fecal coliform counts comparable to those of the elevated temperature, multiple-tube procedure. Other investigators have reported increased fecal coliform recoveries by eliminating rosolic acid from the medium formulation or by simply preincubating m-FC agar plates at 35°C for 5 h before shifting them to 44.5°C for 18 h (6, 11). Based on a physiological approach, Stuart et al. (13) developed an injury-mitigating MF procedure that included glycerol and acetate plus reducing agents in both layers of a two-layer medium. Fecal coliform recoveries were 1.3 to 19 times greater than the standard m-FC recoveries.

In recognition of the problems associated with enumerating injured bacteria, a new section entitled "Stressed Organisms" (section no. 920) was added to the 15th edition of *Standard Methods for the Examination of Water and Wastewater* (1).

Recently, we developed a new medium (m-T7 agar) for the improved recovery of injured total coliforms (7). We observed that analine blue and bile salts, two ingredients in m-FC agar, were very inhibitory to injured coliforms (7, 10). m-T7 agar contains neither of these ingredients and has been shown to be superior to m-Endo agar for the recovery of total coliforms from drinking water. The present study was designed to determine whether m-T7 agar was effective for recovery of injured fecal coliforms as well.

MATERIALS AND METHODS

Sample area and sample collection. Secondary sewage effluents were collected from the chlorinated wet well, contact chamber, or outfall ditch of several sewage treatment plants near Bozeman, Mont., during the midmorning hours (9 to 11 a.m.) of October 1982 through October 1983. These sewage treatment plants received domestic and light industrial waste as well as runoff from a storm water collection system. Effluent-free residual chlorine levels ranged from 0 to 0.6 mg/liter (average 0.24 mg/liter); and total chlorine levels ranged from 0.14 to 0.7 mg/liter (average 0.34 mg/liter). Effluent temperatures ranged from 9 to 20°C (average 13.6°C), and pH ranged from 6.3 to 7.5 (average 7.1). In addition, samples were collected from a sewage lagoon which served a residential area near Bozeman. Sewage in this lagoon was not chlorinated but was characterized by an alkaline pH (9.2).

Samples were collected in sterile 2-liter polypropylene bottles containing 0.008% (final concentration) sodium thiosulfate (1). Chlorine residuals were determined on site by the *N,N*,diethyl-*p*-phenylenediamine colorimetric method with a Hach model CN-70 chlorine test kit (Hach Chemical Co., Loveland, Colo.). Samples were placed on ice and transported to the laboratory within 1 h. A preliminary test was done for some samples to determine the approximate fecal coliform level. In these cases, the samples were stored at 4°C overnight. All samples were analyzed within 30 h after collection.

Microbiological analyses. Media comparisons were made by filtering three replicates of each sample dilution through Millipore HC type filters (Millipore Corp., Bedford, Mass.), using the following six MF techniques. The standard fecal coliform procedure and two resuscitation techniques were performed according to *Standard Methods* (sections 909C

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and 920) (1). One resuscitation technique consisted of eliminating rosolic acid from the m-FC medium. The other resuscitation method was similar to the two-layer enrichment, temperature acclimation procedure of Rose et al. (12). These three techniques were labeled m-FC S (standard), m-FC R^- (without rosolic acid), and m-FC LA (m-FC agar with a lauryl tryptose agar overlay and incubation for 4 h at 35°C followed by 20 h at 44.5°C). Because fecal coliform recoveries were optimal at 4 h of preincubation on m-FC agar (see Fig. 1), this time was used for the media comparisons rather than the 2 h recommended by *Standard Methods* (1). m-T7 agar (7) was used as the fecal coliform medium in the next three techniques. One method consisted of simply incubating the medium for 24 h at 44.5°C (labeled m-T7 44.5°C). The other two techniques involved preincubating the plates for 8 h at 35°C (labeled m-T7 8 h 35°C) or 37°C (labeled m-T7 8 h 37°C) followed by incubation for an additional 12 h at 44.5°C.

All smooth yellow convex colonies on m-T7 agar or blue colonies on m-FC agar were counted with the aid of a dissecting microscope (×15 to ×20). At least 10 presumptive fecal coliforms from each technique and sample were verified by inoculation into lauryl tryptose broth (35°C for 48 h) followed by transfer to EC broth (44.5°C for 24 h). Fecal coliforms were identified by the API-20E system (Analytab Products, Plainview, N.Y.)

Media and materials. m-FC broth base (lot no. 689377) supplemented with 1.5% agar (lot no. 686162 and 704723), lauryl tryptose broth (lot no. 685074), and EC broth (lot no. 701122) were purchased from Difco Laboratories, Detroit, Mich. Rosolic acid (lot no. 81F-0007) was obtained from Sigma Chemical Co., St. Louis, Mo. m-T7 agar (containing 0.1 μg of penicillin G per ml) was prepared as previously published (7). A heat sink incubator and a circulating water bath, both capable of achieving 44.5 ± 0.2°C, were used for the elevated temperature tests.

Quality control and statistical comparisons. A quality assurance program as outlined in *Standard Methods* (1) was used throughout the course of this study. Performance of media and sterility controls were determined on a per lot or a per batch basis. Materials used during each experiment were checked for sterility. The temperatures of autoclaves and incubators were monitored on a per use basis.

Because widely varying dilutions (1 to 10^{-4} ml) were used for different water samples, counts were tabulated on a per filter basis and were represented irrespective of dilution. Statistical comparisons were made on logarithmically transformed data by the paired Student's *t* test.

RESULTS

Temperature is recognized as a major selective factor in the fecal coliform procedure. As a result, experiments were designed to determine the optimum preincubation time and temperature for m-T7 agar. Plates were preincubated at 35°C for up to 8 h before transfer to 44.5°C (total incubation time for all plates was 24 h) (Fig. 1). Results indicated only a small increase in fecal coliform recoveries during the first 4 h, but a dramatic increase (3.43-fold) in fecal coliform recoveries was observed with a preincubation time of 8 h. Similar increases were seen for m-FC agar, but after 4 h at 35°C many small, blue, nonfecal coliform colonies also appeared. Because these small, typical, blue colonies did not verify as fecal coliforms, it was not possible to preincubate m-FC agar longer than 4 h without the sensitivity of the medium being reduced. With m-T7 agar, nonfecal coliform colonies did not appear with 8 h of preincubation. Although it may be possible to recover more fecal coliforms with even longer preincubation times, nonfecal coliform colonies will also occur. For this reason, the 8-h preincubation interval is an optimum trade-off between selectivity and sensitivity with m-T7 agar.

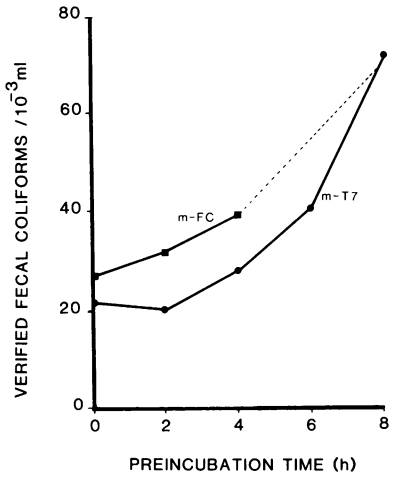

FIG. 1. Effect of preincubation time on fecal coliform recovery. Plates were incubated up to 8 h at 35°C followed by incubation at 44.5°C. Total incubation time for all plates was 24 h. Increases in fecal coliform recovery on m-FC agar were observed after 4 h of preincubation; however, many small, blue, noncoliform colonies also were observed after this time.

To determine the optimum temperature, m-T7 agar plates were preincubated for 8 h at various temperatures, including 25, 32, 35, and 37°C (Fig. 2). The highest fecal coliform recoveries were observed by preincubating m-T7 agar plates at 37°C for 8 h. Isolation rates for fecal coliforms at 37°C (8 h) were 32% higher than fecal coliform recoveries at 35 or 32°C, 68% higher than recovery rates at 25°C, and nearly 10 times the isolation rates for fecal coliforms without any preincubation (Fig. 2). Combinations of incubation times and

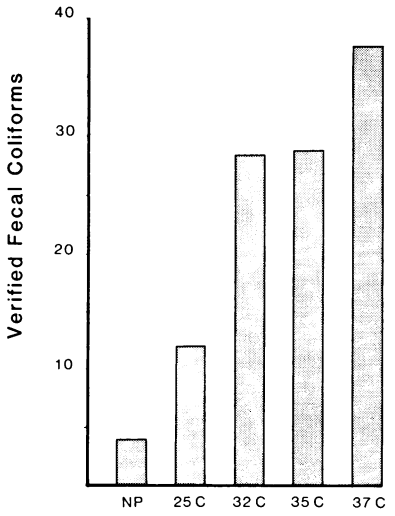

FIG. 2. Comparison of preincubation temperature for fecal coliform recovery on m-T7 agar. Plates were incubated at the designated temperature for 8 h followed by 16 h at 44.5°C. NP, No preincubation.

TABLE 1. Comparison of m-FC and m-T7 techniques

Date	Site	pH	Temp (°C)	Free chlorine (mg/liter)	Total chlorine (mg/liter)	Dilution factor (per 100 ml)	No. of verified fecal coliforms per filter by the following technique:[a]					
							m-T7 44.5°C	m-FC S	m-FC R^-	m-FC LA	m-T7 8 h 35°C	m-T7 8 h 37°C
10/11/82	Outfall no. 1	6.9	9.0	0.2	0.3	4.0×10^4	10.2	12.5	14.4	20.3	27.8	ND[b]
10/11/82	Wet well no. 1	6.7	10.0	0.6	0.7	4.0×10^4	5.1	11.2	9.0	15.7	18.4	ND
10/18/82	Outfall no. 1	7.2	11.0	0.2	0.3	4.0×10^4	12.6	13.6	25.6	38.7	36.0	ND
10/18/82	Wet well no. 1	7.2	10.0	0.2	0.5	4.0×10^4	16.0	22.5	29.0	37.3	80.6	ND
10/25/82	Outfall no. 1	7.5	11.5	0.2	0.2	4.0×10^4	5.1	7.6	12.8	46.0	83.3	ND
10/25/82	Wet well no. 1	7.5	11.0	0.2	0.2	4.0×10^4	12.0	14.5	23.7	24.7	53.9	ND
12/6/82	Contact chamber no. 1	7.2	16.0	0.3	0.5	5.0×10^3	12.6	8.5	24.3	55.2	ND	70.5
12/13/82	Contact chamber no. 1	7.2	11.0	0.2	0.2	5.0×10^3	3.9	5.6	8.1	10.8	16.8	25.6
1/3/83	Contact chamber no. 1	7.1	9.0	0.0	0.1	1.0×10^3	6.0	17.1	17.4	21.6	40.3	47.0
1/10/83	Contact chamber no. 1	6.3	11.0	0.1	0.3	6.7×10^1	8.6	7.2	16.8	33.0	30.1	39.0
1/24/83	Contact chamber no. 1	6.9	9.0	0.4	0.5	1.6×10^1	2.0	6.7	7.4	22.6	23.0	28.3
9/8/83	Outfall no. 1	6.8	17.0	0.0	0.0	2.0×10^2	2.3	10.8	17.8	15.6	17.7	49.0
9/9/83	Outfall no. 2	7.0	18.0	0.0	0.0	3.3×10^3	6.7	19.2	14.1	19.4	26.7	23.4
9/12/83	Outfall no. 1	7.3	18.0	0.0	0.0	1.0×10^2	12.0	33.0	39.6	39.9	34.8	40.5
9/12/83	Lagoon	9.2	18.0	NA[c]	NA	1.0×10^2	0.3	5.8	8.0	7.5	10.3	12.7
9/14/83	Outfall no. 3	7.2	20.0	0.2	0.3	1.0×10^2	1.7	5.4	7.5	15.3	14.2	18.9
9/19/83	Outfall no. 1	7.0	15.0	0.0	0.0	3.3×10^3	2.3	9.0	12.0	11.7	13.0	16.8
9/20/83	Outfall no. 2	7.1	15.0	0.0	0.0	1.0×10^2	3.1	8.7	13.7	13.4	11.4	32.0
10/4/83	Outfall no. 1	7.1	17.0	0.0	0.0	3.3×10^3	13.5	22.5	26.4	12.2	25.0	32.2
10/4/83	Lagoon	9.2	15.0	NA	NA	3.3×10^2	6.8	12.8	19.2	25.2	55.0	68.7

[a] See the text for a full description of procedures.
[b] ND, Not done.
[c] NA, Not applicable.

temperatures (e.g., 25°C for 4 h and then 35°C for 4 h followed by 44.5°C for 16 h) did not improve fecal coliform recoveries above the 37°C (8 h) level. Temperatures higher than 37°C were not tested because it was thought that these elevated temperatures would aggravate the cellular lesion (6, 13, 14).

Repeated analyses indicated that fecal coliform counts did not significantly increase after a 20-h total incubation time (data not shown); rather, colonies on m-T7 agar incubated for a full 24 h produced so much acid that neighboring nonfecal coliform colonies started to take on a yellowish appearance. The difference between fecal and nonfecal coliform colonies was much more distinct when plates were incubated for a total time of 20 h.

Fecal coliform counts for six techniques tested on 20 samples of secondary sewage effluent are given in Table 1. Recovery of fecal coliforms by the m-T7 8-h 37°C technique was significantly greater ($P < 0.001$) than each of the m-FC procedures tested. The m-T7 8-h 37°C method recovered an average of 30.1 verified fecal coliforms per filter, whereas m-T7 8-h 35°C recovered 21.7; m-FC LA, 17.2; m-FC R^-, 14.1; m-FC S, 10.6; and m-T7 44.5°C, 3.7 (Fig. 3). Recoveries by the m-T7 8-h 37°C technique were an average 3.1 times greater than the standard m-FC method and 1.7 times greater than the m-FC LA procedure. When parallel analyses of m-T7 8-h 35°C and m-T7 8-h 37°C were done (13 samples), the m-T7 8-h 37°C method resulted in a 39.2% increase in fecal coliform recoveries.

Verification rates for the three m-T7 agar techniques were 93.3% (169 of 181) for m-T7 44.5°C, 83.2% (158 of 190) for m-T7 8-h 35°C, and 90.7% (127 of 140) for m-T7 8-h 37°C (overall average 89.0%). Slightly lower verification rates were observed for the m-FC procedures: 77.5% (155 of 200) for m-FC S, 87.4% (174 of 199) for m-FC R^-, and 83.2% (158 of 190) for m-FC LA (overall average, 82.8%).

Background bacterial counts averaged from nine samples (collected from all locations) are presented in Table 2. The ratio of average background count (per filter) to the average fecal coliform count (per filter) specifies the number of background colonies per fecal coliform isolated. The data in Table 2 indicate that the m-T7 8-h 37°C technique had nearly the same ratio of background bacteria to fecal coliforms as did the m-FC procedures. The data show that it is possible to isolate 60 fecal coliforms by the m-T7 8-h 37°C procedure

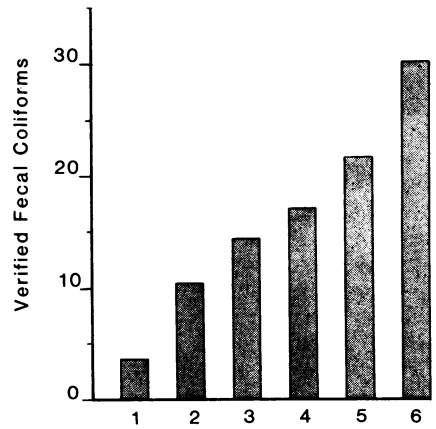

FIG. 3. Comparison of fecal coliform recoveries from 13 chlorinated sewage effluent samples. Columns: 1, m-T7 44.5°C; 2, m-FC S; 3, m-FC R^-; 4, m-FC LA; 5, m-T7 8 h 35°C; 6, m-T7 8 h 37°C. See the text for a further description of techniques.

TABLE 2. Background bacterial counts on fecal coliform media

Technique[a]	Avg background count[b]	Background to coliform ratio[b]
m-T7 44.5°C	0.9	0.17
m-FC S	3.1	0.30
m-FC R^-	3.7	0.25
m-FC LA	7.1	0.38
m-T7 8 h 35°C	40.22	1.49
m-T7 8 h 37°C	18.2	0.57

[a] See the text for an explanation of techniques.
[b] Average background counts are tabulated on a per filter basis.
[c] Ratio is calculated by the following formula: ratio = average background count per average fecal coliform level.

and not exceed 100 colonies on the filter. In addition, the results show that nearly 3 times less background bacteria appeared on plates preincubated at 37°C than at 35°C. The difference in background bacterial populations growing at 35 and 37°C was not further investigated.

Identification of 60 fecal coliforms isolated by the m-FC LA and m-T7 8-h 35°C procedures revealed that the two media recovered nearly the same coliform populations. *E. coli* was the predominant fecal coliform on both media, comprising 70% of the isolates on m-T7 agar and 63.3% of the isolates on m-FC medium. *Klebsiella pneumonia* totalled 26.7 and 33.3% of the isolates on m-T7 and m-FC agar, respectively. *Enterobacter agglomerans* and *Citrobacter freundii* were isolated once as fecal coliforms on m-T7 and m-FC agars, respectively.

DISCUSSION

In this study, m-T7 agar was superior to all other methods tested when preincubated at 37°C for 8 h before transfer to 44.5°C for 20 h. This new technique recovered an average of 3.1 times more fecal coliforms than the standard m-FC method and 1.7 times more than the m-FC LA procedure. The reason for the improved recovery is twofold: (i) use of a medium specifically designed to improve recoveries of injured coliforms, and (ii) adequate preincubation time at an optimum temperature to allow for improved recovery of stressed coliforms.

Before the development of m-T7 medium, we surveyed over 20 different media commonly used for coliform analysis (10). The majority of selective media used to isolate gram-negative bacteria recovered 30% or less of the injured coliforms. This induced recovery was related to the concentration of deoxycholate and bile salts in the media. Currently, all media formulations for fecal coliform enumeration, except m-T7 agar, contain deoxycholate or bile salts as a selective ingredient (5, 6, 9, 11–13). In addition, subsequent studies demonstrated that aniline blue, the differential agent in m-FC medium, was highly toxic to injured coliforms (7). By examining each ingredient in the m-T7 formulation for optimal recovery of laboratory-injured cells, we were assured of a highly efficient medium for the isolation and enumeration of coliforms from environments in which injury is an important consideration.

Results from this study have confirmed previous reports that temperature is a major inhibitory factor in the fecal coliform procedure when injured cells are being isolated. Accurate fecal coliform enumerations are not possible without adequate preincubation time to allow for complete resuscitation of stressed cells. Our data suggest that 8 h at 37°C is of value in the recovery of fecal coliforms. With this preincubation time, significant increases in isolation rate were observed for both m-FC and m-T7 media. However, numerous small nonfecal coliforms were apparent on m-FC agar, whereas these organisms were not seen on m-T7 plates. It was because of these nonfecal coliform colonies that m-FC medium was limited to only 4 h of preincubation. m-T7 agar remained selective even with an 8-h preincubation time. The overall verification rate for m-T7 agar was 89%, and the medium recovered the same fecal coliform population as the m-FC LA technique.

Results of this study indicate that 66.8% of the coliforms in the chlorinated sewage effluents examined were injured (as determined by the difference between the m-T7 8-h 37°C and m-FC S techniques). Previous laboratory studies have indicated the cell envelope as the major site of chlorine damage (4, 14). Experiments have shown that respiration, glucose transport, and ATP levels all decrease in injured populations (4). Electron microscopy of injured cells has demonstrated morphological changes in the cell membrane (14). These reports indicated that repair of injured membranes usually occurred 3 to 4 h after resuscitation. In this study, optimal recovery of fecal coliforms from chlorinated sewage effluents did not occur until after an 8-h resuscitation period. It is unknown what physiological process requires an 8-h adaptation interval, but the data suggest that the resuscitation time for bacteria injured in the environment may be longer than previously thought.

Several advantages are immediately apparent in having this one medium capable of performing both the total and fecal coliform tests. (i) The medium is simple. There are no overlay procedures, nor does the filter need to be transferred. (ii) The medium is convenient. Since contamination of water supplies is usually transitory, it is neither expedient nor convenient to prepare another medium to assay for fecal pollution. In an emergency, it would be easier to incubate the same medium at an elevated temperature for the fecal coliform test. (iii) Finally, use of one medium for two tests could save money. Costs could be cut by not having to purchase a variety of media and chemicals and by saving time in media preparation.

One disadvantage is that an incubator is required that is capable of adjusting temperatures after an 8-h interval. Although the technology is readily available and a similar device is on the market (Millipore Corp., Bedford, Mass.), we are not aware of such an incubator designed specifically for this method. In addition, the proposed two-temperature fecal coliform test has a problem when successive sets of samples are processed during the day. Such logistical problems can be overcome by several temperature-adjusting incubators. The initial cost of acquiring these incubators is offset by the increased sensitivity and accuracy of the m-T7 8-h 37°C technique.

The results of this study have shown that the m-T7 8-h 37°C technique was superior to all other methods tested for the recovery of fecal coliforms from sewage effluents. The procedure had high fecal coliform recoveries, high verification rates (90.7%), and a low number of background colonies (0.57 CFU per fecal coliform). Additional testing of the m-T7 8-h 37°C method needs to be done in other geographical areas to fully evaluate the effectiveness of this procedure for the recovery of injured fecal coliforms from sewage.

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