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Modification of M-FC Medium by Eliminating Rosolic Acid

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Eliminating rosolic acid from M-FC medium improves the MFC procedure by allowing higher fecal coliform colony recoveries with greater ease in counting. Samples of unchlorinated and chlorinated domestic sewage, creek, lake, and river water were analyzed for fecal coliforms by standard procedures. Results of 200 comparisons of fecal coliform counts on M-FC medium without and with rosolic acid showed that higher counts were obtained 71% of the time when rosolic acid was excluded without an overgrowth of background colonies. Results from analyzing chlorinated sewage showed that eliminating rosolic acid improved the recovery of fecal coliform bacteria by 49%. A total of 1,675 blue colonies and 766 nonblue colonies were verified. Of the 1,675 blue colonies, 1,566 were confirmed as fecal coliform bacteria, for a verification of 93.5%. The percent verification of nonblue colonies as noncoliform bacteria was 84.2% (644/766).

Selective recovery of fecal coliform bacteria is achieved either by specific chemicals in the culture medium or by increasing the incubation temperature above the optimum for growth. In 1904 Eijkman (4) discovered that an incubation temperature of 46°C was selective for recovering *Bacillus coli* (*Escherichia coli*). Since then, various investigators have used incubation temperatures of 41°C (D. J. Van Donsel, R. M. Twedt, and E. E. Geldreich, Bacteriol. Proc., p. 25, 1969), 42°C(7), 43°C(3), 44°C(11), 44.5°C(5), 45°C(6), and 45.5°C(9) for selective recovery of fecal coliform bacteria. The MFC procedure as given in *Standard Methods* (1) specifies the use of both chemical additives and an elevated temperature of 44.5°C for enumerating fecal coliform organisms. According to Geldreich et al. (5), the sodium salt of rosolic acid in M-FC medium suppresses more of the nonfecal bacteria from certain water sources. Similarly, rosolic acid is recommended to inhibit the growth of nonfecal bacteria that may grow at the elevated temperature after the first runoff after a rainfall (12).

We found that when we eliminated rosolic acid from M-FC medium, fecal coliform colonies were an intense royal blue on membrane filters. The vivid blue color of the colonies made counting of fecal coliform colonies easier than when rosolic acid was used because of the sharper contrast between fecal and nonfecal colonies. To see whether we could eliminate rosolic acid from M-FC medium without an overgrowth of nonfecal bacteria, we compared fecal coliform recoveries from a variety of water sources on membrane filters by using M-FC media without and with rosolic acid.

MATERIALS AND METHODS

Samples. Samples were collected over a 5-month period from lake, creek, river, and domestic sewage. Lake Lila is a small lake with a permanent duck population and is subject to significant runoff after rainfall. The Honey Creek sampling site is a fast-flowing stream receiving agricultural runoff. Three sampling sites were chosen on the Huron River. Site 1 receives industrial effluents from several small industries. Sites 2 and 3 are located away from industries and sewage treatment plants. Ann Arbor and Loch Alpine sewage treatment plants receive only domestic sewage.

Chlorinated sewage. Domestic sewage was chlorinated in our laboratory with calcium hypochlorite (Olin HTH). Chlorine in concentrations of 2.1 to 2.4 mg of total chlorine per liter as determined by the iodometric method (1) was added to 100 ml of raw sewage. The samples were shaken intermittently. After a 15- to 20-min exposure of the samples to chlorine at ambient temperature, the chlorine was neutralized with 1 ml of 0.1% sodium thiosulfate. Analysis was begun immediately. The percent kill of the coliform population achieved was 95 to 99, calculated from the reduction in cell numbers compared with untreated sewage.

Media. M-FC medium (Difco Laboratories) was prepared in 500-ml Erlenmeyer flasks. After heating to boiling, the medium was divided into two equal portions. To one portion was added 0.01% rosolic acid. Rosolic acid (Difco) was prepared every 3 days by dissolving 1 g of the sodium salt of rosolic acid in 100 ml of 0.2 N NaOH. Storage was at 4°C. The other portion was used as M-FC broth without rosolic acid.

Analysis of samples. Analysis of the samples was done by the membrane filter technique of *Standard Methods* (1). Membrane filters (Gelman Metricel GN-6, Millipore HC, and Sartorius SM 114) were used in this study. However, the purpose of this study was not

to compare commercial brands of membrane filters but to determine if fecal coliform bacteria produce typical blue colonies on each brand of filter with M-FC medium without rosolic acid. Five replicates with each brand of filter were run.

Confirmation of colonies. To confirm fecal coliform bacteria typical blue colonies were chosen at random from membrane filter cultures on M-FC medium without rosolic acid. Nonblue colonies were picked to confirm noncoliform bacteria. Well-isolated colonies were inoculated into phenol red lactose broth and were incubated at 35°C. Cultures producing gas after 24 and 48 h were inoculated into EC broth. EC cultures were incubated at 44.5°C for 24 h. Cultures producing gas in EC broth were recorded as fecal coliform bacteria.

Analysis of data. Data were analyzed by the analysis of variance, correlation analysis, and linear regression analysis. The linear regression equation used was $Y = bX$, where Y represents the dependent variable and X represents the independent variable. The letter b is the estimate of the regression coefficient obtained by least squares.

RESULTS

The results of colony counts of fecal coliform bacteria on M-FC media without and with 0.01% rosolic acid are given in Table 1. Mean fecal coliform colony counts for all water sources were higher when rosolic acid was eliminated, with the exception of Huron River site 3. The results were not significantly higher at this site when rosolic acid was present in the medium.

Data from the analysis of variance showed no significant differences in the internal variation between colony counts of M-FC media with or without rosolic acid. Correlation coefficients, calculated from the means of the colony counts, showed that M-FC media without and with rosolic acid were measuring the same parameter, fecal coliform bacteria. As shown in Table 1, correlation coefficients for colony counts from both types of media are near unity, which is excellent correlation for results between the two media.

Coliform bacteria may be nonlethally injured or physiologically stressed upon exposure to chlorine, temperature extremes, and various chemicals. Results for regression analysis data for chlorinated sewage and site 1 of Huron River are shown in Fig. 1. The Huron River site is located below the city of Ann Arbor and receives effluents from industrial operations. The line of equality (Fig. 1) represents perfect agreement

TABLE 1. *Colony counts of fecal coliform bacteria and correlation coefficients on M-FC media without and with rosolic acid*

Sample source[a]	No. of runs	Mean of fecal coliforms/100 ml		Correlation coefficient
		WO/RA[b]	W/RA[c]	
Ann Arbor sewage	78	38.0	35.1	0.983
Loch Alpine sewage	10	54.3	51.9	0.974
Chlorinated sewage	32	81.6	54.7	0.968
Huron River				
Site 1	10	26.0	23.9	0.989
Site 2	22	42.9	39.6	0.986
Site 3	12	28.2	28.4	0.968
Honey Creek	18	46.1	44.0	0.995
Lake Lila	18	46.0	42.6	0.973

[a] Samples were collected after rainfall in many instances.
[b] WO/RA, Without rosolic acid
[c] W/RA, With 0.01% rosolic acid.

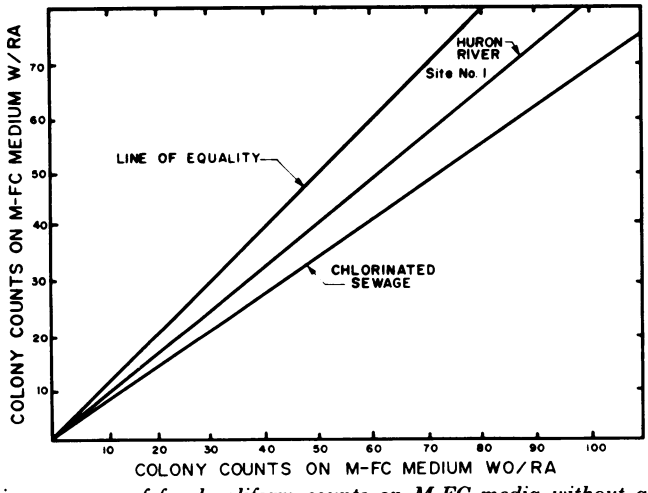

FIG. 1. *Comparative recovery of fecal coliform counts on M-FC media without and with rosolic acid. Standard error of the regression coefficient is 0.04 for Huron River and 0.03 for chlorinated sewage.*

between colony counts on M-FC medium without and with rosolic acid. The distance between the line of equality and the regression line represents the difference in results between the two media. These results suggest that significantly higher counts of physiologically stressed fecal coliform bacteria are recovered on M-FC medium when rosolic acid is excluded. Numerically, an average of 49% more colonies of fecal coliform bacteria were recovered from chlorinated sewage and 9% more colonies from Huron River water on M-FC medium without rosolic acid. The results are statistically significant at the 95% confidence level.

Total colony background counts on M-FC medium without rosolic acid. Because the purpose of rosolic acid in M-FC medium is to suppress the noncoliform bacterial flora, total colony counts were compared on M-FC media without and with 0.01% rosolic acid. The results shown in Table 2 compare the means of the total colony counts at 35 and 44.5°C. With the exception of samples 1 to 8 from the Ann Arbor sewage plant, all samples were collected after rainfall. The reason for using 35°C was to find the maximum number of bacteria in the sample that will grow on M-FC medium. The colony counts at 35°C were compared with those at 44.5°C to see the effects of the elevated temperature upon the development of nonfecal bacteria. Comparison of colony counts at 35°C on M-FC media in the presence and absence of rosolic acid shows that a higher number of colonies develop in the absence of rosolic acid. Therefore, rosolic acid at this temperature is inhibitory for some species of bacteria. Results of total colony counts at 44.5°C, however, show no significant differences between total colony counts of un-

TABLE 2. *Comparison of total colony counts on M-FC media at 35 and 44.5°C*[a]

Source of sample	Total colony counts at:				
	35°C		44.5°C		
	W/RA[b]	WO/RA[c]	W/RA	WO/RA	(WO/RA)/(W/RA)
Ann Arbor sewage	330,000,000	490,000,000	500,000	900,000	1.80
	1,740,000,000	2,200,000,000	1,200,000	1,100,000	0.92
	1,940,000,000	2,200,000,000	1,200,000	1,200,000	1.00
	170,000,000	280,000,000	870,000	1,200,000	1.38
	90,000,000	120,000,000	1,100,000	1,300,000	1.18
	85,000,000	65,000,000	2,000,000	2,100,000	1.05
	50,000,000	80,000,000	1,600,000	1,700,000	1.06
	63,000,000	77,000,000	1,600,000	1,600,000	1.00
Ann Arbor sewage[d]	180,000,000	180,000,000	1,900,000	2,100,000	1.11
	120,000,000	180,000,000	1,700,000	1,800,000	1.06
Huron River	60,800	79,800	350	310	0.89
Site 2[d]	48,400	55,600	330	350	1.06
	29,400	36,600	130	130	1.00
	184,200	211,000	200	230	1.15
Site 3[d]	20,000	28,000	260	260	1.00
	15,000	25,000	330	370	1.12
	10,000	13,000	340	340	1.00
	71,400	87,200	240	250	1.04
	51,600	68,400	340	310	0.91
	43,400	54,400	140	140	1.00
	28,800	34,400	170	170	1.00
	212,800	200,800	190	180	0.95
Honey Creek[d]	30,000	48,600	380	370	0.97
	31,000	26,200	350	360	1.03
	59,000	85,000	700	700	1.00
Lake Lila[d]	28,500	58,200	810	840	1.04
	44,800	57,000	790	850	1.08

[a] Results based on number of colonies per 100 ml.
[b] W/RA, M-FC medium with 0.01% rosolic acid.
[c] WO/RA, M-FC medium without rosolic acid.
[d] Samples collected after rainfall.

stressed bacteria on the two types of media. The ratios of total colony counts without rosolic acid to the counts with rosolic acid at 44.5°C have a range of 0.89 to 1.80 with a mean of 1.07. These results indicate that the elevated temperature, not rosolic acid, is responsible for inhibition of growth of the noncoliform bacteria. The elevated temperature caused a 244-fold decrease in the total colony of 35°C when rosolic acid was used. There was a 274-fold decrease in total colonies at the elevated temperature when rosolic acid was eliminated.

Verification of fecal coliform colonies. Most fecal coliform colonies on M-FC medium without rosolic acid are distinctly blue, but some bacterial species produce blue colonies with a white periphery. Others are clear with a definite blue center. However, all colonies are easily distinguishable from noncoliform bacterial colonies, which are cream and grey.

A total of 1,675 blue colonies and 766 nonblue colonies were verified. Of the 1,675 blue colonies, 1,566 were confirmed as fecal coliform bacteria for a verification of 93.5%. The percent verification of nonblue colonies as noncoliform bacteria was 84.2% (644/766).

DISCUSSION

Results of this study covering the analyses of 200 samples from a variety of sources suggest that the recovery of fecal coliform bacteria is enhanced when rosolic acid is eliminated from M-FC medium. The reason for the improved recovery of fecal coliform bacteria without an overgrowth of nonfecal bacteria is the ability of fecal coliform bacteria to grow at elevated temperatures. Thermotolerance is a characteristic of fecal coliform bacteria that enables these bacteria to proliferate at elevated temperatures of 41 to 46°C. The ability to grow at elevated temperatures facilitates selective recovery of fecal coliform bacteria. Other enteric bacteria are not equally competitive with fecal coliform bacteria at these incubation temperatures. The few species of fecal bacteria capable of growth at 44 to 44.5°C (the most commonly used incubation temperatures) infrequently ferment lactose and thereby are incapable of producing false coliform reactions. Some species of thermophilic lactose-fermenting gram-positive bacilli are capable of growth at the elevated temperature, but their occurrence is infrequent in water analysis.

Selective chemical compounds such as bile salts and dyes in culture media aid in selective enumeration of specific groups of bacteria. Bronfenbrenner et al. (2) suggested rosolic acid as a selective agent for the isolation of intestinal bacteria at 37.5°C. Following their suggestion, Geldreich et al. (5) added rosolic acid to M-FC medium.

Our results show that rosolic acid in M-FC medium has an adverse effect on the nonfecal population of bacteria at 35°C. However, at an elevated incubation temperature of 44.5°C, total colony counts on M-FC medium without rosolic acid were not higher than those on M-FC medium with 0.01% rosolic acid, whereas fecal coliform counts were always higher at the elevated temperature. These results suggest that inhibition of the elevated temperature masks any inhibitory effect rosolic acid has on limiting the background bacterial flora.

Although rosolic acid apparently does not affect normal cells of fecal coliform bacteria, our results from analyzing chlorinated sewage show that development of nonlethally injured fecal coliform bacteria is affected by rosolic acid. These findings of adverse effects by chemicals used in culture media for metabolically stressed bacteria agree with the results of others. Scheusner et al. (10) found that crystal violet, neutral red, and brilliant green were inhibitory for the recovery of *E. coli* exposed to sodium hypochlorite. Rosolic acid, crystal violet, neutral red, and brilliant green belong to the triphenylmethane group of dyes and share common chemical properties. Maxey (8) reported that injury of bacteria by chlorine was one of the environmental stresses that hinders recovery of organisms on media containing selective agents.

Modifying M-FC medium by eliminating rosolic acid improves the recovery of fecal coliform bacteria and facilitates the counting of colonies. The increase in percent recovery is statistically significant for metabolically stressed coliform bacteria. The percent verification of all blue colonies is slightly higher on M-FC medium without rosolic acid than with it. The results reported here do not indicate that rosolic acid is necessary for preventing an overgrowth of nonfecal bacteria after runoff from rainfall. The elevated temperature is not limiting to the growth of thermotolerant fecal coliforms on M-FC medium while inhibiting the development of noncoliforms. Water sources where rosolic acid is recommended certainly are the exception and not the usual water samples analyzed by the M-FC procedure. The rare occurrence of such samples does not justify routine use of rosolic acid. Using rosolic acid is expensive in material costs and labor. Rosolic acid varies in its chemical purity, which increases the complexity of a complex system. A footnote on p. 894 of the 14th edition of *Standard Methods* (1) states that rosolic acid may be eliminated from M-FC medium if results indicate it is not needed. We recommend that M-FC medium be used without

rosolic acid; if in rare instances it is needed, then use it only with those samples.

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