Delayed-Incubation Membrane-Filter Test for Fecal Coliforms

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A delayed-incubation membrane-filter technique for fecal coliforms was developed and compared with the immediate fecal coliform test described in Standard Methods for the Examination of Water and Wastewater (13th ed., 1971). Laboratory and field evaluations demonstrated that the delayed-incubation test, with the use of the proposed vitamin-free Casitone holding medium, produces fecal coliform counts which very closely approximate those from the immediate test, regardless of the source or type of fresh-water sample. Limited testing indicated that the method is not as effective when used with saline waters. The delayed-incubation membrane-filter test will be especially useful in survey monitoring or emergency situations when the standard immediate fecal coliform test cannot be performed at or near the sample site or when time and temperature limitations for water sample storage cannot be met. The procedure can also be used for analyzing the bacterial quality of water or waste discharges by a standardized procedure in a central examining laboratory remote from the sample source.

There is a growing recognition and use of the fecal coliform group as an indicator of recent fecal contamination of surface waters (2, 5). Enumeration of these organisms provides a more precise measure of the potential health hazard associated with recreational waters and raw water supplies than does the more ubiquitous total coliform group. For this reason, both the Report of the National Technical Advisory Committee on Water Quality Criteria (4) and Geldreich (6) recommended that fecal coliforms be used as the indicator group for evaluating the microbiological suitability of recreation waters. The Ohio River Valley Sanitation Commission water users committee (11) recommended the use of fecal coliforms for measuring the bacterial quality of raw water supplies, and Geldreich and Bordner (7) suggested the use of this parameter for measuring the quality of irrigation waters and the degree of disease hazard caused by pathogen occurrence on fruits and vegetables.

Widespread use of fecal coliform criteria in water pollution surveillance has been hindered by the lack of a delayed-incubation membrane-filter procedure comparable to the delayed membrane-filter method for total coliforms. The 13th edition of Standard Methods for the Examination of Water and Wastewater (1) recommended that the bacteriological examination of raw samples be initiated within 1 h (or within 8 h if iced), because the extended storage of water samples generally results in unpredictable changes in bacterial counts (3, 9, 10, 13, 14). The delayed-incubation total coliform procedure is recommended when the above time and temperature limitations cannot be met. A delayed-incubation fecal coliform procedure, however, has not been available. This paper presents a new technique and a new medium that fill this definite need in the quantitation of fecal coliforms.

A procedure of this type would eliminate the need for a field incubator and would free the field investigator from the time-consuming task of counting colonies. Also, examination at a central laboratory would permit confirmation and complete biochemical identification of the organisms if desired.

The development of a delayed test for fecal coliforms was dependent upon formulation of a holding medium which would meet the following requirements: (i) delayed fecal coliform counts must closely approximate the immediate count regardless of the source or type of water sample; (ii) all microbial growth must be
inhibited for a 3-day holding period (the maximal first-class mailing time between any two points in the United States); (iii) there must be no carry-over of growth-suppressive material after transferral of the held membrane to a selective growth medium; (iv) acceptable results must be obtained in the wide range of ambient temperatures that are encountered under differing geographical and seasonal conditions; and (v) the holding medium formulation should be simple enough to be easily prepared and readily available in the average microbiological laboratory.

A transport medium for fecal coliforms was reported in England by Panezai, Macklin, and Coles (12) for use in the evaluation of creamery wastes. This medium was composed of 0.02% peptone-water containing 0.4% sodium benzoate as a preservative agent. However, this formulation did not prove satisfactory as a holding medium in studies conducted in this laboratory. Possible reasons for this failure are the higher temperatures and longer shipping times encountered in the United States as compared with those in the United Kingdom.

MATERIALS AND METHODS

Holding medium development. The peptone derivatives evaluated as minimal nutrient base components in a transport medium for fecal coliforms included Casitone, Pantone, Peptone, Proteose Peptone No. 3, Protone, Soytone, Thiotone, tryptone, vitamin-free Casamino Acids, and vitamin-free Casitone (VFC). These nutrient bases were used in combination with several inhibiting agents including sodium chloride, ethanol, sulfanilamide, sodium benzoate, and other food preservatives. Modifications of existing specialized media (m-Endo Broth MF, m-Coliform Holding Medium LES, m-FC Broth, Cary and Blair Transport Medium, Transport Medium Stuart) were also evaluated as possible holding media for fecal coliforms.

All but one of the above media proved to be unsatisfactory as fecal coliform holding media, as a result, in most cases, of rapid bacterial growth on the filters, lack of differentiation of fecal coliforms after transferral of the filters to m-FC medium (8), or a combination of these factors.

Only the VFC medium containing sodium benzoate, sulfanilamide, and 95% ethanol (Table 1) gave consistently good results. Because of its low vitamin content and the inhibiting agents used, this medium will not support bacterial growth but will keep cells viable for the required holding period.

Holding medium evaluation. Samples from nine different sampling locations in the Cincinnati, Ohio, area were collected as described in Standard Methods (p. 657) and examined in the laboratory by the standard immediate (IMF) and experimental delayed-incubation (DMF) membrane-filter procedures for fecal coliforms. In the delayed incubation technique, the water sample was filtered through 0.45-μm membrane filters, and the filters were placed in tight-lid plastic petri dishes containing absorbent pads saturated with 2.0 ml of the VFC holding medium. These delayed membrane filters were retained in a constant temperature box at 25 ± 2°C (approximating room temperature) for 1, 2, or 3 days to simulate transport conditions. Five replicate filtrations were performed for each test.

After the predetermined storage period, filters were removed from the VFC holding medium and placed in tight-lid plastic petri dishes containing absorbent pads saturated with m-FC broth, a selective growth medium for fecal coliforms (8). The dishes were placed in water-proof plastic bags and incubated submerged in a water bath at 44.5°C for 22 ± 2 h. After this incubation period, typical fecal coliform colonies were counted under a binocular dissecting microscope at 15 times magnification. The average delayed fecal coliform count (DMF) per 100 ml was then computed and compared to the immediate count (IMF) to provide a ratio, DMF/IMF, which would ideally be 1.00.

After the laboratory evaluations of the holding medium were completed, a field evaluation was conducted in cooperation with Environmental Protection Agency laboratories located at Wheeling, W.Va., Edison, N.J., Denver, Colo., and Evansville, Ind. The participating laboratories performed IMF and DMF fecal coliform determinations in triplicate on water samples from a variety of sources.

The delayed test membranes were placed on the VFC holding medium and mailed to the Analytical Quality Control Laboratory in Cincinnati. Upon receipt in Cincinnati, the delayed filters were transferred to m-FC broth and incubated as previously described for the laboratory evaluations. The resultant fecal coliform colonies were counted and a DMF/IMF ratio was established. This interlaboratory evaluation determined the effectiveness of the VFC holding medium on waters with wide ranges of quality and geographical distribution.

RESULTS AND DISCUSSION

Table 2 shows the DMF/IMF ratios for the 1-, 2-, and 3-day delayed fecal coliform tests.

| Ingredient          | Amt     |
|---------------------|---------|
| Vitamin-free Casitone | 0.02 g  |
| Sodium benzoate     | 0.40 g  |
| Sulfanilamide       | 0.05 g  |
| Ethanol (95%)       | 1.00 ml |
| Final pH            | 6.7     |

* Warm to dissolve ingredients; then sterilize medium by filtration through 0.22-μm membrane filter.  
* Use 2.00 ml of a 1:100 vitamin-free Casitone aqueous solution.

Table 1. Vitamin-free Casitone holding medium* (ingredients per 100 ml)
## Table 2. Ratios comparing immediate (IMF) and delayed (DMF) fecal coliform counts from the Cincinnati area

| Sample                  | Date*      | Immediate count/100 ml* | 1-day DMF/IMF | 2-day DMF/IMF | 3-day DMF/IMF |
|-------------------------|------------|-------------------------|---------------|---------------|---------------|
| Ohio River mile 462.2   | 9-4-69     | 2,000                   | 0.90          | 1.60          | —             |
|                         | 2-17-70    | 330                     | 0.85          | 0.91          | 0.67          |
| Ohio River mile 467.5   | 9-23-69    | 1,800                   | 1.00          | 1.06          | 1.61          |
|                         | 10-29-69   | 12,000                  | 1.00          | 0.92          | 0.81          |
|                         | 11-4-69    | 12,000                  | 1.67          | 1.58          | 1.74          |
|                         | 5-13-70    | 1,800                   | 0.83          | 0.65          | 1.22          |
|                         | 7-9-70     | 560                     | 0.80          | 0.69          | 0.65          |
|                         | 9-1-70     | 11,000                  | 0.47          | 0.47          | 1.09          |
| Ohio River mile 470.2   | 7-1-69     | 3,000                   | 0.83          | 0.87          | 1.10          |
|                         | 7-8-69     | 6,800                   | 0.81          | 0.81          | 0.90          |
|                         | 7-15-69    | 1,000                   | 0.56          | 0.65          | 0.88          |
|                         | 7-18-69    | 540                     | 1.17          | 0.67          | 0.96          |
|                         | 7-23-69    | 4,700                   | 0.89          | 1.02          | 0.87          |
|                         | 8-19-69    | 11,000                  | 0.62          | 0.54          | 0.65          |
|                         | 9-19-69    | 1,000                   | 2.50          | 3.10          | 3.60          |
|                         | 12-9-69    | 1,500                   | 0.93          | 1.00          | 1.33          |
|                         | 12-16-69   | 440                     | 0.73          | 0.82          | 1.05          |
|                         | 1-13-70    | 5,000                   | 0.80          | 0.88          | 0.56          |
|                         | 3-24-70    | 600                     | 0.82          | 0.93          | 1.07          |
|                         | 4-15-70    | 700                     | 0.77          | 0.73          | 0.66          |
|                         | 4-21-70    | 1,100                   | 0.64          | 0.47          | 0.31          |
|                         | 12-1-70    | 1,700                   | 0.82          | 1.54          | 1.64          |
| Ohio River mile 477.5   | 9-30-69    | 3,000                   | 0.87          | 0.63          | —             |
|                         | 10-7-69    | 4,200                   | 1.02          | 0.93          | 1.00          |
|                         | 10-14-69   | 5,100                   | 0.76          | 0.80          | 1.63          |
|                         | 1-27-70    | 10,000                  | 0.52          | 0.44          | 0.50          |
|                         | 1-29-70    | 1,900                   | 1.37          | 0.95          | 1.05          |
|                         | 5-5-70     | 6,800                   | 0.96          | 1.04          | 1.35          |
|                         | 5-14-70    | 47,000                  | 0.68          | 0.92          | 0.47          |
| Burnet Woods Lake       | 8-19-69    | 1,500                   | 0.93          | 0.87          | —             |
| Little Miami River mile 1.5 | 12-2-69   | 2,600                   | 1.42          | 1.42          | 1.62          |
|                         | 2-4-70     | 2,000                   | 1.30          | 1.80          | 1.70          |
|                         | 3-10-70    | 840                     | 0.57          | 0.30          | 0.26          |
|                         | 3-24-70    | 2,800                   | 0.71          | 0.39          | 0.39          |
| Five Mile Creek         | 12-2-69    | 610                     | 0.75          | 0.56          | 0.69          |
| Licking River mile 0.3  | 1-7-70     | 1,800                   | 0.89          | 0.83          | 1.06          |
|                         | 2-10-70    | 590                     | 0.81          | 0.81          | 1.32          |
| Mill Creek              | 10-13-70   | 2,800,000               | 1.40          | 1.43          | 1.64          |

* Each count is an average of five replicate filtrations.

* Month-day-year.

that were performed in the laboratory on 38 water samples from the Cincinnati area. The immediate counts per 100 ml ranged from 330 fecal coliforms/100 ml at Ohio River mile 462.2 upstream from Cincinnati to highs of 47,000/100 ml at river mile 477.5 downstream from Cincinnati and 2,800,000/100 ml in Mill Creek. Each count per 100 ml is an average of five replicate filtrations. Missing values were the result of confluent bacterial growth on the 3-day delayed filters which made colony counting impossible. Standard Methods (p. 684) stipulates that acceptable fecal coliform counts be limited to 20 to 60 colonies per membrane.
Counts outside this range may result in errors due to statistical inaccuracy, sampling variations, crowding, or colony confluence on the membrane filter.

Table 3 lists the mean, variance, standard error of the mean, and 95% confidence limits for the data contained in Table 2. The mean DMF/IMF ratios indicate that on the average the delayed fecal coliform counts closely approximate the immediate counts. The 1- and 2-day delayed counts averaged slightly lower and the 3-day counts slightly higher than the initial counts. The 95% confidence limits of the mean increased progressively as the holding period increased.

For verification as fecal coliforms, 1,475 “typical” blue colonies from the above samples were picked from the immediate, 1-, 2-, and 3-day delayed membranes and inoculated first into lactose broth and then into EC medium. The confirmation in EC medium was excellent (95%; Table 4) and demonstrated that holding the membrane filters on VFC holding medium in no way affected the response of the fecal coliforms on the m-FC broth.

Tables 5 and 6 show the results of the field studies conducted in cooperation with Environmental Protection Agency laboratories in Evansville, Wheeling, Edison, and Denver. Samples of both high and low bacterial density showed good DMF/IMF correlation in these studies. Differences in transit times had no demonstrable effect on the delayed counts.

The DMF/IMF ratios for the Edison and Wheeling area samples showed lower correlation than those from Evansville or Cincinnati. Special characteristics of the Edison and Wheeling samples could have affected fecal coliform recovery. More erratic results from the Wheeling area may have been due to suspected toxicity from acid mine drainage and steel mill effluents. Toxic substances retained on the filter surface or physiologically weak bacteria present in the stream, or both, could account for the lower delayed counts. Also, the correct dilution to give between 20 and 60 colonies per filter was not always obtained from these samples. Consequently, it was necessary to use counts of greater than 60 colonies per filter in some cases. Even though this 60-colony limitation was on occasion exceeded, individual colonies were easily countable with no evidence of confluency or overcrowding. The Edison samples, taken from the estuarine waters of Raritan Bay, generally had delayed counts that were significantly higher than the immediate counts. If the VFC holding medium is to be used in saline waters, further evaluations of its effectiveness with these waters should be undertaken first.

In summary, each of these evaluations was designed to determine the effectiveness of the VFC holding medium under varying laboratory and field use conditions, representing wide geographical, seasonal, and water quality ranges. The data show that inoculated membrane filters can be held on the VFC holding medium for 1 to 3 days with little effect on the fecal coliform counts. The minimal holding period and the use of air mail or special delivery, or both, when applicable are recommended. Use of the described delayed-incubation test is suggested only when the standard immediate fecal coliform test cannot be performed.

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### Table 5. Comparison of immediate and delayed fecal coliform counts from various geographical regions

| Sample                                | Date*  | Count/100 ml | Ratio, D/I | Time in transit (h) |
|---------------------------------------|--------|--------------|------------|---------------------|
|                                       |        | Immediate (I) | Delayed (D) |                     |
| Area of Evansville, Ind.              |        |              |            |                     |
| Ohio River mile 803.5                 | 7-22-70| 350          | 450        | 1.29                | 26                   |
| Ohio River mile 841.5                 | 8-3-70 | 340          | 470        | 1.38                | 19                   |
| Ohio River mile 827.3                 | 8-1-70 | 510          | 650        | 1.10                | 46                   |
| Ohio River mile 616.6                 | 10-13-70| 27,000       | 22,000     | 0.81                | 18                   |
| Ohio River mile 791.5                 | 10-14-70| 2,600        | 1,800      | 0.69                | 24                   |
| Green River                           | 11-18-70| 280          | 240        | 0.86                | 44                   |
| Wabash River                          | 11-18-70| 660          | 960        | 1.45                | 44                   |
| Ohio River mile 791.5                 | 12-9-70 | 1,100        | 1,000      | 0.91                | 19                   |
| Ohio River mile 803.5                 | 12-9-70 | 1,100        | 1,200      | 1.09                | 18                   |
| Wabash River                          | 12-9-70 | 540          | 550        | 1.02                | 24                   |
| Ohio River mile 646.0                 | 12-15-70| 4,800        | 5,100      | 1.06                | 68                   |
| Ohio River mile 616.6                 | 12-15-70| 30,000       | 29,000     | 0.97                | 67                   |
| Area of Wheeling, W. Va.              |        |              |            |                     |
| Ohio River at Warwood                 | 9-23-70 | 180          | 130        | 0.72                | 21                   |
| Wheeling Creek                        | 10-7-70 | 8,600        | 4,700      | 0.55                | 45                   |
| Ohio River at Old L&D 19              | 10-12-70| 140          | 91         | 0.65                | 44                   |
| Ohio River at Warwood                 | 10-28-70| 150          | 140        | 0.93                | 29                   |
| Ohio River at Warwood                 | 11-3-70 | 570          | 350        | 0.61                | 24                   |
| Big Sandy River                       | 11-18-70| 1,300        | 1,100      | 0.85                | 28                   |
| Wheeling Creek                        | 11-23-70| 980          | 600        | 0.61                | 22                   |
| Ohio River at Warwood                 | 12-2-70 | 1,100        | 1,200      | 0.91                | 51                   |
| Ohio River at Warwood                 | 12-8-70 | 940          | 590        | 0.63                | 27                   |
| Wheeling Creek                        | 12-9-70 | 710          | 480        | 0.68                | 30                   |
| Ohio River at South Hts               | 12-14-70| 850          | 770        | 0.91                | 49                   |
| Ohio River at Marietta                | 12-14-70| 670          | 580        | 0.87                | 49                   |
| Wheeling Creek                        | 12-21-70| 780          | 180        | 0.73                | 49                   |
| Mill Acres                            | 9-8-71  | 340          | 500        | 1.45                | 20                   |
| Ohio River at Old L&D 19              | 9-14-71 | 1,400        | 1,900      | 1.35                | 24                   |
| Ohio River at Ashland                 | 9-15-71 | 1,500        | 1,400      | 0.93                | 48                   |
| Big Sandy River                       | 9-15-71 | 210          | 270        | 1.28                | 24                   |
| Wheeling Creek                        | 9-22-71 | 290          | 220        | 0.75                | 24                   |
| Area of Edison, N.J. (estuarine waters)|        |              |            |                     |
| Seawaren                              | 9-21-71 | 9,000        | 8,900      | 0.98                | 50                   |
| Perth Amboy                           | 9-23-71 | 300          | 700        | 2.33                | 50                   |
| Perth Amboy                           | 9-28-71 | 780          | 510        | 0.65                | 53                   |
| Perth Amboy                           | 10-4-71 | 120          | 280        | 2.33                | 23                   |
| Seawaren                              | 10-4-71 | 1,700        | 4,000      | 2.34                | 23                   |
| Seawaren                              | 10-5-71 | 8,000        | 9,900      | 1.23                | 48                   |
| Perth Amboy                           | 10-5-71 | 460          | 770        | 1.67                | 48                   |
| Perth Amboy                           | 11-16-71| 200          | 220        | 1.10                | 28                   |
| Seawaren                              | 11-16-71| 3,400        | 4,200      | 1.23                | 28                   |
| Area of Denver, Colo.                 |        |              |            |                     |
| North Platte                          | 1-11-72 | 2,100        | 2,300      | 1.10                | 44                   |
| St. Vrain                             | 9-7-71  | 860          | 1,200      | 1.40                | 64                   |

*All values based on three replicate counts:

*Month-day-year.

*Approximate value based on colony counts greater than 60.

### Table 6. Statistical comparison of the immediate (IMF) and the delayed (DMF) membrane-filter fecal coliform tests on samples from various geographical regions

| Region                     | Mean ratio, DMF/IMF | 95% confidence limits of the mean | SE of mean | Variance | No. of samples |
|----------------------------|---------------------|----------------------------------|------------|----------|----------------|
| Area of Evansville, Ind.   | 1.05                | 0.92-1.18                        | 0.07       | 0.05     | 12             |
| Area of Wheeling, W. Va.   | 0.84                | 0.70-0.98                        | 0.07       | 0.10     | 18             |
| Area of Edison, N.J.       | 1.54                | 1.12-1.96                        | 0.21       | 0.42     | 9              |
| All areas combined*        | 1.05                | 0.92-1.18                        | 0.05       | 0.21     | 42             |

*These figures include the three Denver area samples.
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