Pfizer Selective Enterococcus Agar Overlay Method for the Enumeration of Fecal Streptococci by Membrane Filtration

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The use of Pfizer selective enterococcus (PSE) agar with the membrane filter technique for the enumeration of fecal streptococci is limited due to the inability of the characteristic black precipitate, indicative of esculin hydrolysis, to diffuse from the medium through the membrane. A modification of the membrane filter technique that consisted of placing the membrane on PSE agar and overlaying it with tempered PSE agar was evaluated by comparing recovery, selectivity, and other parameters with M-enterococcus and KF-streptococcus agars, two selective media routinely used with the membrane filter technique for the enumeration of fecal streptococci in water and wastewater. No statistically significant differences could be demonstrated in the recovery capabilities of the three media. Inasmuch as the PSE overlay technique requires only 24 h of incubation as opposed to 48 h for the other two media, this modification may have some merit in water pollution monitoring programs.

The enumeration of fecal streptococci from water and wastewater employing the standard membrane filter (MF) technique requires 48 h of incubation. In 1970 Isenberg et al., using Pfizer selective enterococcus (PSE) agar, demonstrated that these microorganisms could be isolated and identified from clinical specimens within 24 h (4). Pavlova et al. confirmed this in a comparative study that included the enumeration of fecal streptococci from water, soil, food, and feces by employing the PSE direct plating technique (5). During this study, however, it was concluded that the use of PSE with the membrane filter technique was limited due to the inability of the characteristic black precipitate, indicative of esculin hydrolysis, to diffuse from the medium through the membrane. To determine whether the colonies that develop on the membrane hydrolyze esculin, a modification of the membrane filter technique was suggested that consisted of placing the membrane on PSE agar and overlaying it with tempered PSE agar. The “sandwiched” membrane was incubated at 35 C for 24 h. The PSE overlay technique was evaluated by comparing recovery, selectivity, ease of preparation, and use with M-enterococcus (ME) and KF-streptococcus (KF) agar, two selective media routinely used to enumerate fecal streptococci in water and wastewater.

MATERIALS AND METHODS

Sampling sites. River, lake, and marine water samples of varying levels of pollution were collected during the winter and spring of 1974. Most sampling stations were within 10 miles (ca. 16 km) from the laboratory; the marine samples, however, were collected from a Long Island beach known to be polluted.

Primary and secondary effluents were collected from sewage treatment plants in Amherst, South Deerfield, and North Brookfield, Mass. Samples were generally collected between 12 and 2 p.m., which represented the peak flow of most effluent discharges. In addition, fecal specimens from cows, sheep, horses, rabbits, chickens, and geese were obtained from the University farm. These were collected in sterile paper cartons, whereas water and sewage samples were collected in wide-mouth 300-ml sterile glass sample bottles.

Bacteriological analyses of all samples were initiated within 1 h after collection with the exception of the marine water, which required 3 to 4 h enroute.

Media preparation. ME (BBL), KF (BBL), and PSE (Pfizer) agars were prepared according to the manufacturers' directions.

Approximately 5 to 6 ml of each medium was aseptically pipetted into polystyrene plastic petri dishes (60 by 15 mm; Millipore Corp.). The media were allowed to cool and solidify at room temperature. These plates were usually used within 8 h of preparation; however, when surpluses were encountered they were stored in plastic air-tight pouches at 4 C for no longer than 1 week.

Filtration. Appropriate sample volumes were analyzed to obtain counts of between 20 and 100 colonies per filter (HA-047-AO, 0.45 μm, Millipore Corp., Bedford, Mass.). In the case of most water samples, these volumes ranged from 100 to 0.1 ml, whereas
sewage was serially diluted to $10^{-4}$ in phosphate buffer (pH 7.2). Fecal specimens were likewise serially diluted to $10^{-4}$; however, glass beads were added to the first bottle of each dilution series to facilitate uniform suspension of the fecal material. Generally, 4 volumes of each sample were filtered to insure countable plates. Unless otherwise stated, all methods employed conformed to *Standard Methods for the Examination of Water and Wastewater* (1).

After filtration, the membranes were aseptically transferred to petri dishes that contained the selective agar media. In the case of PSE plates, an overlay of 1.5 to 2.0 ml of liquid agar, tempered to 45 to 50 °C, was added so that the filter was sandwiched between PSE layers. Upon solidification of the overlay, PSE plates were inverted and incubated at 35 °C for 24 h; KF and ME plates were incubated for 48 h.

**Colony enumeration.** Colonies from all three media were counted after 24 h by employing a wide-field binocular stereoscope ($\times 15$) equipped with the proper illumination. ME and KF plates were reincubated and counted again after an additional 24 h. Pink to dark red colonies of varying size were indicative of fecal streptococci on ME and KF agar, whereas only tan to black colonies surrounded by the characteristic dark brown to black zones were counted in the PSE overlay.

**Colony identification.** A representative number of typical colonies from the types listed in Table 1 from all MPs was randomly verified and speciated. This number was derived by computing the square root of colonies enumerated; however, at least eight colonies were confirmed from each plate. Isolated surface colonies were picked from membranes on ME and KF plates, whereas subsurface colonies in PSE were stabbed through the overlay.

Colonies selected for confirmation were grown in brain heart infusion (Difco) broth for 24 h at 35 °C. The broth cultures were checked for purity by streaking onto Trypticase soy agar (BBL) plates which were then incubated at 35 °C for 18 to 24 h. All catalase-negative isolates were subjected to: (i) preliminary verification and (ii) identification according to the schemes of Pavlova et al. (5).

**RESULTS**

A comparison of fecal streptococcal recovery employing the MF technique with PSE, KF, and ME agars was conducted on 39 water, sewage, and fecal samples. All colonies enumerated were categorized into various groups based on colony morphology (Table 1).

Geometric means of fecal streptococci recovered from fresh water, marine water, animal feces, and sewage effluent are listed in Tables 2 through 5, respectively.

The data in Table 2 demonstrate that counts on KF agar were at least 20% higher than those on the PSE overlay from 8 of 14 river samples. PSE agar, on the other hand, yielded higher counts from 12 of 14 river samples after 24 h of incubation than did ME agar after 48 h. Moreover, these results indicate that fecal streptococci were recovered by use of the PSE overlay from both relatively clean and polluted river sites (5 and 6) as effectively as when KF was used but in half the time. Although differences in fecal streptococci recovery between KF agar and the PSE overlay were demonstrated at river sites 1 through 4 and at surface water sites 8 and 9, statistical analysis of the data failed to demonstrate any significant differences between these two media.

In contrast to fresh water samples, the highest counts from marine water samples were demonstrated with the PSE overlay (Table 3). Similarly, counts obtained from animal feces were highest on the PSE overlay (Table 4). The counts on ME agar were similar to those on KF agar from all animal fecal samples except geese.

### Table 1. Description of fecal streptococci colony types on the test media

| Colony type | PSE                                      | ME                                 | KF                                      |
|-------------|------------------------------------------|------------------------------------|-----------------------------------------|
| I           | On filter, tan to brown, round, entire, zone of iron salts; 2 mm or more* | Dark red to maroon with or without pink margin; 2 mm or more | Dark red to maroon with or without pink margin; 2 mm or more |
| II          | On filter, tan to brown, round, entire, zone of iron salts; 0.5-2.0 mm | Dark red to maroon with or without pink margin; 0.5-2.0 mm | Dark red to maroon with or without pink margin; 0.5-2.0 mm |
| III         | On filter, color variable, minute faint zone of iron salts; 0.5 mm | Minute pink to red granular center; 0.5 mm | Minute pink to red granular center; 0.5 mm |
| IV          | On surface of agar with zone of iron salts, size variable | Light pink to pink; 0.5-2.0 mm | Light pink to pink; 0.5-2.0 mm |

* Diameter.
TABLE 2. Geometric means of fecal streptococci recovered from river, lake, and pond samples on the test media after 24- and 48-h incubation periods

| Water source     | No. of samples | Density* |
|------------------|----------------|----------|
|                  |                | PSE (24 h) | ME (24 h) | KF (24 h) | ME (48 h) | KF (48 h) |
| Connecticut River| Site 1         | 2         | 6,762     | 1,058     | 2,737     | 5,413     | 10,711     |
|                  | Site 2         | 3         | 1,341     | 238       | 659       | 679       | 1,998      |
|                  | Site 3         | 3         | 537       | 172       | 332       | 693       | 1,141      |
|                  | Site 4         | 1         | 1,300     | 220       | 600       | 600       | 1,900      |
| Mill River       | Site 5         | 3         | 6,061     | 319       | 1,925     | 2,203     | 5,676      |
|                  | Site 6         | 1         | 75        | 5         | 10        | 15        | 85         |
| Fort River       | Site 7         | 1         | 31        | 8         | 14        | 20        | 22         |
| Mt. Warner Lake  | Site 8         | 3         | 476       | 198       | 608       | 338       | 615        |
| Campus pond      | Site 9         | 1         | 2,300     | 850       | 1,100     | 1,200     | 3,200      |

*Number per 100 ml of water.

TABLE 3. Geometric means of fecal streptococci recovered from marine samples on the test media after 24- and 48-h incubation periods

| Sample no. | Density* |
|------------|----------|
|            | PSE      | ME       | KF       |
|            | (24 h)   | 24 h     | 48 h     | 24 h     | 48 h     |
| 1          | 130      | 45       | 190      | 120      | 140      |
| 2          | 105      | 60       | 100      | 52       | 68       |
| 3          | 165      | 56       | 65       | 74       | 130      |

*Number per 100 ml of water.

where the KF yield was considerably higher than ME. Likewise, extremely high fecal streptococci counts were obtained from geese feces with the PSE overlay when compared to the other media.

The highest recovery of fecal streptococci from sewage was demonstrated with KF agar (Table 5); however, as with the river water data, no significant differences could be demonstrated between KF and the PSE overlay upon statistical analyses of the data.

A total of 876 colonies was subjected to preliminary verification of fecal streptococci (5). Table 6 shows the number and percentage of the major groups isolated on the three selective media. Also shown are the number and percentage of nonfecal streptococci and catalase-positive colonies that appeared as typical colonies on each medium. The data indicate that between 92 and 95% of the isolates recovered and examined from the test media were fecal streptococci and demonstrate the selectivity of these media. Moreover, these media supported a relatively low percentage of nonfecal streptococci and catalase-positive isolates having typical fecal streptococci characteristics.

Table 7 shows the number and percentage of enterococcal species examined and identified from the test media. A total of 267 cultures was examined by the identification scheme of Pavlova et al. (5). Table 8 lists the various biotypes isolated from each selective medium. These data also show the corresponding precipitin reaction for each biotype. Precipitin reactions were indicated as immediate or delayed positive reactions. A majority (70%) of all randomly selected biotypes was found to carry the group D antigen. Biotypes 5 and 9 were predominantly recovered on ME and KF agars from the South Deerfield sewage treatment plant.

Analyses of variance were conducted on mean densities of fecal streptococci. The analyses indicated that a significant difference in the recovery of fecal streptococci from fresh water and sewage samples existed \( F = 3.19 \) for fresh water and \( F = 3.31 \) for sewage, whereas no significant difference was shown from sea water and fecal specimens. Further statistical comparisons (q contrasts) indicated that the only
TABLE 4. Geometric means of fecal streptococci recovered from animal fecal samples on the test media after 24- and 48-h incubation periods

| Fecal source | No. of samples | Density* |
|--------------|----------------|----------|
|              |                | PSE (24 h) | ME 24 h | ME 48 h | KF 24 h | KF 48 h |
| Cow          | 3              | 7.9 x 10^4 | 2.3 x 10^4 | 5.0 x 10^4 | 2.3 x 10^4 | 3.2 x 10^4 |
| Sheep        | 2              | 8.7 x 10^4 | 2.4 x 10^4 | 3.8 x 10^4 | 3.1 x 10^4 | 3.4 x 10^4 |
| Horse        | 1              | 5.5 x 10^4 | 1.9 x 10^4 | 2.4 x 10^4 | 2.4 x 10^4 | 4.0 x 10^4 |
| Rabbit       | 2              | 6.9 x 10^4 | 2.4 x 10^4 | 4.2 x 10^4 | 4.9 x 10^4 | 4.9 x 10^4 |
| Chicken      | 1              | 5.3 x 10^4 | 3.0 x 10^4 | 2.0 x 10^4 | 3.2 x 10^4 | 3.3 x 10^4 |
| Geese        | 1              | 5.0 x 10^4 | 1.0 x 10^4 | 3.0 x 10^4 | 2.0 x 10^4 | 5.2 x 10^4 |

* Number per gram of feces.

TABLE 5. Geometric means of fecal streptococci recovered from sewage samples on the test media after 24- and 48-h incubation periods

| Sewage source | No. of samples | Density* |
|---------------|----------------|----------|
|               |                | PSE (24 h) | ME 24 h | ME 48 h | KF 24 h | KF 48 h |
| Amberst       | 3              | 8.9 x 10^4 | 5.4 x 10^4 | 3.6 x 10^4 | 6.2 x 10^4 | 1.2 x 10^4 |
| North Brookfield | 2          | 2.8 x 10^4 | 9.0 x 10^4 | 1.5 x 10^4 | 2.6 x 10^4 | 2.9 x 10^4 |
| South Deerfield | 3            | 6.3 x 10^4 | 1.5 x 10^4 | 5.6 x 10^4 | 7.1 x 10^4 | 4.8 x 10^4 |

* Number per 100 ml of sample.

TABLE 6. Number and percentage of colonies verified from the test media

| Group         | PSE | ME | KF |
|---------------|-----|----|----|
|               | No. | %  | No. | %  | No. | %  |
| Enterococci   | 212 | 68.39 | 203 | 77.78 | 162 | 59.11 |
| Streptococcus | 39  | 12.58 | 19  | 7.28  | 35  | 11.48 |
| bovis         |     |      |    |       |     |     |
| Strep. equinus| 5   | 1.61 | 0   | 0.00  | 2   | 0.66 |
| Enterococcal  | 23  | 7.42 | 26  | 9.96  | 72  | 23.60 |
| biotypes      |     |      |    |       |     |     |
| S. salivarius | 15  | 4.84 | 1   | 0.38  | 12  | 3.83 |
| Total fecal   | 294 | 94.84 | 249 | 95.40 | 283 | 92.78 |
| strepto-      |     |      |    |       |     |     |
| cocci         |     |      |    |       |     |     |
| Nonfetal      | 15  | 4.84 | 11  | 4.21  | 19  | 6.23 |
| strepto-      |     |      |    |       |     |     |
| cocci         | 1   | 0.32 | 1   | 0.38  | 3   | 0.98 |

TABLE 7. Number and percentage of enterococci species identified from the test media

| Enterococcal species | PSE | ME | KF |
|----------------------|-----|----|----|
|                      | No. | %  | No. | %  | No. | %  |
| S. faecalis and var. | 21  | 22.8| 42  | 45.2| 27  | 33.0|
| S. faecium           | 34  | 37.0| 20  | 21.5| 23  | 28.0|
| S. durans            | 32  | 34.8| 22  | 23.6| 29  | 35.4|
| Other intermediates  | 5   | 5.4 | 9   | 9.7 | 3   | 3.6 |

DISCUSSION

From the data it can be concluded that ME agar yielded lower recoveries of fecal streptococci than did PSE or KF agar. It was determined that this medium could not support the growth of the viridans streptococci, which may be a factor in the determination of the pollutant's origin. On the other hand, ME agar was highly selective for the isolation of the enterococci group, an observation first reported by Slanetz and Bartley (9) and later by Sureau (10) and Croft (2). Conversely, reports by Ribaudo et al. and Saraswat et al. demonstrated that Lactobacillus sp. grow readily on ME agar (6, 8), but this was not demonstrated in the present study.
Excellent fecal streptococci recovery from water and sewage samples was demonstrated on KF agar. However, when animal feces were examined, it was found that these organisms were recovered in higher numbers on PSE agar after 24 h of incubation than on KF agar after 48 h. As a result of this observation, the use of KF agar for the examination of waters that receive substantial amounts of animal wastes warrants reevaluation. These findings tend to substantiate those of Raibaud et al., who reported low recoveries of *Streptococcus bovis* with KF agar and observed that AGAT medium yielded 100 times the KF counts when fecal samples from pigs were examined (6).

The observation that PSE agar yielded higher counts from domestic fowl feces, especially from geese, suggests that this medium may support the growth of *S. avium*. This group Q streptococcus warrants consideration in the determination of the sanitary quality of water and wastewater. Work is now in progress to determine whether, in fact, *S. avium* was the predominant species from these samples.

In this study, KF agar provided the highest recovery of fecal streptococci from sewage but also allowed an excessive diversity of biotypes to develop. Although many of these biotypes possessed the group D antigen, their sanitary significance has not been adequately investigated. The KF agar showed further limitations in recovery of these indicators from marine waters. In addition to its relatively long incubation requirement, KF was limited by the complexity in preparation and the difficulty in enumeration of colonies. Complexity in preparation arises from the fact that a 1% 2,3,5-triphenyl tetrazolium chloride solution must be aseptically added to the tempered rehydrated medium. Enumeration of typical fecal streptococcal colonies on KF agar is complicated by extensive growth of uncharacteristic colonies and frequent diversity among typical colonies on plates with high numbers.

PSE agar, used with the overlay technique, was shown to facilitate recovery of fecal streptococci from various sources while retaining a high degree of selectivity as effectively as the other media that require 24 h of additional incubation. Others, employing conventional methods, found PSE agar to be an effective presumptive isolation medium for fecal streptococci. Isenberg et al. reported PSE agar to be as sensitive as other clinical isolation media (i.e., blood agar and mitis-salivarius agar) and considered its selectivity superior (4). Sabbaj et al. indicated that, although the routinely used clinical presumptive media (*S. faecalis* broth and agar) performed well, PSE had the advantage of selecting out *S. bovis*, required no special incubation temperature, and produced distinctive colonies earlier than other media (7). On the other hand, Facklam reported that group D streptococci from human specimens could be differentiated more accurately on bile esculin and modified bile esculin media than on PSE agar (3).

Although the present data indicate that the PSE agar overlay method was highly selective for the recovery of fecal streptococci from water, sewage, and feces and that it is capable of providing presumptive membrane filter results in the same time as is required for coliform and fecal coliform determinations, this method cannot be used in the field unless a portable laboratory is available. Liquid PSE agar, tempered to 45 to 50 C, is necessary for overlaying the membrane filter.

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