Membrane Filter-Fluorescent-Antibody Method for Detection and Enumeration of Bacteria in Water

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A technique which employs nonfluorescing membrane filters and specific fluorescein isothiocyanate–labeled antiserum has been successfully used in the identification and enumeration of known species of Escherichia coli which have been added to natural populations of bacteria found in water. The quantitative results compared favorably with those of standard tests. The use of a dissecting microscope with an external lighting arrangement provided a simple requirement for equipment. This method may be useful in monitoring specific bacterial types from waters which were being monitored for specific pollution.

A method is described for the combined use of membrane filter and fluorescent-antibody techniques to detect and quantitate bacteria in water. The method has been tested as a means of detecting the presence of Escherichia coli in water supplies and has been compared with standard methods with respect to sensitivity and time required. Limitations and the potential of the method to rapidly detect pollution of water supplies have been discussed.

The use of membrane filters with differential media preparations which allow direct specific determination of coliforms has been well established as a means to determine sanitary quality of water (1). In general, these methods require 18 to 24 hr for quantitative counts.

Fluorescent-antibody techniques have been used for the identification of microorganisms from various sources. These techniques, however, require precise microscopic methods, particularly when dark-field microscopy is used. Danielsson (4) and Danielsson and Laurell (4a) reported a membrane-filter method for the demonstration of bacteria by fluorescent-antibody techniques. That method employed filters mounted under the high-power objectives of a microscope, a method requiring manipulation of the membranes and, for quantitation, mathematical calculation. Carter and Leise (3) reported a similar method for enumeration of anthrax colonies.

In water pollution, more rapid detection and enumeration of specific contaminating organisms may be of prime importance. For this reason, our study was initiated to make use of the advantages of the membrane-filter methods for concentration and growth of bacteria and fluorescent-antibody techniques for rapid identification and enumeration of bacteria with the use of low-power, large-field microscopy.

MATERIALS AND METHODS

Production of antiserum. Antiserum to 11 strains of E. coli was produced in rabbits essentially by the methods described by Edwards and Ewing (5). Serum was used to identify specific E. coli strains or selectively pooled to give a polyvalent serum.

Conjugation procedure. The sera were conjugated with fluorescein isothiocyanate by the methods described by Spendlove (8), and further purified after conjugation by means of a diethylaminoethyl cellulose column. The final conjugate was checked for purity and absence of albumin by cellulose acetate strip electrophoresis. Serum was stored at -15 C in stocks of 10 mg of protein per ml. Protein determinations were made by the method of Lowry et al. (6). The stocks were thawed and diluted to a protein concentration of 1 mg/ml prior to use.

Filter equipment. Black, gridded filters (HABG 047, Millipore Corp., Bedford, Mass.) in glass filter bases and funnels were used for this study. White filters and filters from other sources autofluoresce under the conditions of illumination and mask the specific fluorescence.

Fluorescence microscopy. Dark-field preparations were observed with a Nikon SKE microscope (Nikon Inc., Garden City, N.J.) fitted with an ultradark-field condenser (NA 1.30). Illumination was provided by a Bausch and Lomb Illuminator (Bausch & Lomb, Inc., Rochester, N.Y.) with a 200-w mercury arc lamp. Green, neutral-density filters were used for illumination with visible light, and Corning 5-58 or 7-60 filters were used for ultraviolet illumination. Barrier filters were either the appropriate Y-8 or T-2, or the yellow filter supplied with the Nikon equipment. These filter combinations are equivalent to the
count of colonies

Finally, were membranes through procedures previously outlined (9), with no evidence of E. coli pollution. Predominant organisms were Bacillus species and Flavobacterium species.

Water was filtered through the membrane and the filter was transferred to Tryptcase Soy Agar (BBL) to allow growth of the bacteria at 35°C. Colonies could be observed as early as 5 hr; however, for standardization of this method, routine incubation time was 12 hr.

At 12 hr, the filter was placed back on the filter-funnel apparatus. The membrane was overlaid with 1 to 2 ml of pooled normal rabbit serum for 5 min at room temperature. This serum was removed through the membrane by applying negative pressure. Colonies were then overlaid with specific labeled antiserum and allowed to remain in contact with the serum for 15 to 20 min. Labeled serum was pulled through the filter by negative pressure; the colonies were first washed gently with 10 to 15 ml of PBS and finally with glycerol mounting fluid (pH 9.5). The membranes were observed immediately by means of a dissecting microscope (10× magnification); total counts were made under visible light, followed by a count of colonies fluorescing with a green color under ultraviolet illumination.

RESULTS

By use of the techniques described above, experiments were designed to test the efficiency of the MFFA method to accurately detect the presence of varying numbers of fecal E. coli in water samples. To 100 ml of pond water, 10⁹ E. coli ATCC 11303 cells were added. To a separate 100-ml sample, the same number of nonfecal E. coli cells were added. Each preparation was then filtered through separate membranes in amounts of 1.0, 0.5, and 0.1 ml. The membranes were cultured on m-Endo and m-FC (Difco). Trypticase Soy Agar was used for fluorescent-antibody staining. Results are shown in Table 1.

There appears to be little to choose between the methods in efficiency of detection of fecal coliforms in a mixture of organisms in natural waters. The m-Endo and m-FC tests require 18 to 24 hr. These fluorescent-antibody tests were done after 12-hr incubation.

To further test the MFFA method for detection and enumeration of coliforms in water, additional

![Figure 1. Comparison of the accuracy of fecal coliform determination by m-FC culture medium and the MFFA method in relation to the calculated MPN.](http://aem.asm.org/)

**TABLE 1. Comparison of fecal E. coli detection efficiency of three methods**

<table>
<thead>
<tr>
<th>Water sample tested</th>
<th>Amt (ml)</th>
<th>m-Endo</th>
<th>m-FC</th>
<th>MFFA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Green sheen</td>
<td>Total</td>
<td>Blue</td>
<td>Total</td>
</tr>
<tr>
<td>Nonfecal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>0 5 0 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0 7 0 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fecal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>4 134 0 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>17 21 9 10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>28 35 28 29</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values are expressed as number of colonies.

Too numerous to count.
comparisons were made with the use of m-FC medium, and with the most-probable-number (MPN) determination for the water samples (Fig. 1). The results with either method are well within the confidence limits of the calculated MPN.

DISCUSSION

Tests have indicated the reliability and specificity of the MFFA method for detection of fecal coliforms in water supplies. Another advantage of the method is the short time required for completion. Although the work reported here was done at 12 hr for the purpose of standardization of reagents and techniques, additional tests have indicated that the time may be further reduced. For the detection of coliforms, the time required is an extremely important factor which would be of benefit in the testing of water supplies for public health purposes.

In addition, the MFFA method has the advantage of being adaptable to the study of any bacterial species, to use in a variety of pollution studies, and to the study of microbial populations of other interest in water sources.

The adaptation for the use of lower magnification, while requiring somewhat longer than the method of Danielsson (4, 4a), has the advantage of allowing direct counting of the entire filter and eliminates the necessity for calculations. Critical factors in this method are: (i) filters must be washed during staining by pulling solutions through the membrane keeping negative pressure on the filter at all times; (ii) the glycerol mounting fluid must be used to fix the colonies, thus preventing dryness while enhancing fluorescence; and (iii) once exposed to ultraviolet light, the membranes must be counted rapidly before the intense illumination bleaches fluorescence from the stained colonies.

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LITERATURE CITED