Membrane Filter Technique for the Isolation of *Yersinia enterocolitica*

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Received 3 August 1981/Accepted 1 December 1981

A membrane filter procedure was developed for the isolation of *Yersinia enterocolitica* from aquatic environments. Primary differentiation was based on the fermentation of sorbitol, the absence of lysine decarboxylase and arginine decarboxylase-dihydrolase activities, and the production of urease. Sodium deoxycholate was incorporated as an inhibitor of background organisms. The presumptive identification of *Y. enterocolitica* was accomplished in 50 h, and the rate of identity confirmation of typical colonies was 88%. The mean recovery rate of 15 strains from phosphate buffer suspensions was 91%, and quantitative recovery was demonstrated for low populations of the organism in both laboratory-prepared and naturally occurring mixed cultures. The technique was used to isolate 33 strains of *Y. enterocolitica* from 15 of 27 river water samples and from prechlorinated sewage effluent. Nine (27%) of the isolates were rhamnose positive, and only five (15%) were serotypable. Two isolates were identified as serotype O:4 (or O:4,32), two were O:17, and the fifth was O:40.

During the past decade, there have been several widespread outbreaks of human infection with *Yersinia enterocolitica* (2, 3). The documentation of these common-source outbreaks has generated considerable interest in the aquatic environment as a possible reservoir and vehicle of transmission for the disease. Despite numerous reports of the isolation of *Y. enterocolitica* from various aquatic sources (9, 11, 12, 17), there have been few cases in which the presence of the organism has been implicated as the cause of human infection (13, 14). In addition, many aquatic isolates have demonstrated atypical biochemical characteristics, and predominant clinical serotypes have been rarely encountered. These observations have provided a strong argument against the aquatic environment as an important factor in the spread of yersiniosis. However, as Schiemann recently stated (17), the absence of a rapid and reliable method for the recovery of the organism has prevented clarification of the significance of waterborne *Y. enterocolitica*.

The methods currently used for the aquatic isolation of *Y. enterocolitica* have lacked specificity and frequently have required long incubation periods. Common enteric media are often used in attempts to isolate the organism (10, 17). The only differential information provided by most of these media, however, has been the determination of lactose fermentation, and many additional tests must be performed before identifications are made. Cold enrichment techniques have been shown to aid in the recovery of the organism (10), but these methods require several days or weeks of incubation, and the final isolation still relies upon nonspecific plating media.

The purpose of this study was to develop a differential membrane filter technique for the isolation of *Y. enterocolitica*. Such a technique would allow: (i) the sampling of large volumes of water; (ii) a substantial reduction in the number of colonies which must be screened for the isolation of the organism; (iii) a reduction in the amount of time required for isolation and identification; and (iv) reliable recovery of both clinical and environmental strains.

**MATERIALS AND METHODS**

**Cultures.** Fifteen laboratory strains of *Y. enterocolitica* were used for the development of the membrane filter technique for the isolation and presumptive identification of *Y. enterocolitica* (mYE technique). Six strains were provided by James Feeley (Centers for Disease Control, Atlanta, Ga.), and nine were obtained from the culture collection of the Centers for Disease Control in Fort Collins, Colo. (Table 1).

Test suspensions of bacteria were prepared immediately before use by suspending the growth from a 24-h Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) slant in 2 ml of sterile phosphate buffer (SPB), pH 7.2 (1). This suspension was diluted

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in SPB to an optical density of 0.25 at 420 nm, which resulted in a suspension of approximately $5 \times 10^8$ colony-forming units (CFU) per ml. Subsequent dilutions in SPB were prepared as needed.

**mYE technique.** The media and procedure described below for the membrane filter isolation of *Y. enterocolitica* were developed by an empirical trial-and-error study, using known biochemical reactions of the organism and modifications of media described by others.

(i) **mYE recovery broth.** The recovery broth was prepared immediately before use by adding the following compounds (in grams per 100 ml) to deionized water: sorbitol, 1.6; peptone, 0.2; tryptone, 0.2; yeast extract, 0.3; sodium chloride, 0.5; sodium deoxycholate, 0.1; and water-soluble bromothymol blue (Mattheson, Coleman and Bell, Norwood, Ohio), 0.007. The ingredients were dissolved, and the pH was adjusted to 7.0 with 1 N NaOH. The medium was heated with stirring and allowed to boil for 1 min. The mixture was then cooled in an ice-water bath.

(ii) **Lysine-arginine agar.** The lysine-arginine agar assay was a modification of the 4-h lysine decarboxylase test of Brooker et al. (7). The use of lysine and arginine assays in a primary isolation medium for *Y. enterocolitica* was originally proposed by Dudley and Shotts (8). In this study the medium was prepared immediately before use by adding the following compounds (in grams per 100 ml) to deionized water: L-lysine monohydrochloride, 2.0; L-arginine monohydrochloride, 2.0; peptone, 0.3; tryptone, 0.3; yeast extract, 0.5; and agar, 1.5. The ingredients were dissolved and the pH was adjusted to 5.0 to 5.2 with 1 N HCl. After the medium was autoclaved for 15 min at 121°C, it was allowed to cool to 50°C and was dispensed in 6-ml volumes into sterile petri dishes (50 by 120 mm). Before use, plates were preincubated at 35°C for 1 h with lids slightly open.

(iii) **Urease broth.** The urease broth was prepared immediately before use by dissolving 5.0 g of urea in 50 ml of deionized water and mixing with 50 ml of phenol red stock solution. The pH was adjusted to 5.0 with 1 N HCl. The phenol red solution was prepared by dissolving 0.1 g of phenol red in 28.2 ml of 0.01 N NaOH and diluting to 250 ml with deionized water.

The urease broth was a modification of the formulation of Wendt (T. M. Wendt, M.S. thesis, Colorado State University, Fort Collins, 1966). Phenol red was incorporated to provide a more distinct color reaction. The combination of bromothymol blue and phenol red in a membrane filter urease test was originally described by Geldreich and Jeter (E. Geldreich and H. Jeter, Bacteriol. Proc., p. 33, 1952).

Also used in this study were commercially available m-coliform broth (BBL), m-standard methods broth (BBL), and blood agar plates (Pasco Laboratories, Wheatridge, Colo.).

**Evaluation of the mYE technique.** Experiments designed to test the recovery efficiency and accuracy of the technique included both laboratory and field trials.

(i) **Confirmation of colonies.** To verify that the mYE technique could accurately detect the biochemical activities of the bacteria, mixed cultures were prepared and tested. SPB suspensions of 13 gram-negative bacteria were combined with similar suspensions of *Y. enterocolitica*, and the resulting mixtures were serially diluted and plated. The background organisms used in these mixtures were selected so as to provide a diverse challenge to the four biochemical tests of the mYE procedure. Colonies were interpreted either as presumptive *Y. enterocolitica* or as negative and then were picked and identified by conventional tests, as recommended (18). In similar experiments, presumptive positive and negative colonies, selected during the screening of natural water samples, were also identified.

(ii) **Recovery experiments.** The recovery efficiency of the mYE technique was tested by comparing its recovery of SPB suspensions of *Y. enterocolitica* with the recoveries on blood agar spread plates and m-coliform broth membrane filter plates. All plates were incubated at 25°C for 48 h, except for m-coliform broth plates, which were incubated for 72 h. Recovery experiments were also performed by inoculating laboratory-prepared, mixed-culture SPB suspensions and river water samples with various amounts of *Y. enterocolitica*. Samples were then plated according to the mYE procedure, and mYE counts (presumptive positive *Y. enterocolitica*) were determined.

(iii) **Isolation of *Y. enterocolitica* from aquatic sources.** Water samples were collected in sterile plastic bottles according to recommended procedures (1). During the transport, samples were stored on ice, and all samples were processed within 3 h of collection.

Twenty-seven samples were taken from the Cache la Poudre River (Poudre River) in Larimer County, Colo. This stream has been described elsewhere (15). Sampling sites were located in sparsely populated mountain recreation areas and in the plains segment of the river, which passes through agricultural lands and the communities of LaPorte and Fort Collins.

**Serological analysis.** Serotyping tests, performed according to the method of Winblad (19), were conduct-
ed on antigens prepared from each of the 33 cultures isolated during this study. Somatic O antisera prepared in rabbits to 24 serotypes of \textit{Y. enterocolitica} comprised the test battery: O:1-O:9; O:11-O:21; O:11,24; O:4,32; O:40; and O:”Tacoma.” (O:”Tacoma” is a new, undescribed serotype, possessing minor O:26 reactivity, which was originally isolated from a pool of rat fleas collected in Tacoma, Wash.) Antiserum to other serotypes were not available.

**RESULTS**

\textbf{mYE technique.} A flow chart depicting the mYE procedure as developed is presented in Fig. 1. After filtration of the sample, the membrane filter (0.45-μm pore size, 47-mm diameter, type HAWG; Millipore Corp., Bedford, Mass.) was placed on an absorbent cellulose pad (Millipore) saturated with 2 ml of the recovery broth, and the plate was inverted and incubated for 48 h at 25°C. \textit{Y. enterocolitica}, which ferments sorbitol, appeared as bright yellow-to-yellow-orange colonies at this time. The size of the colonies ranged from 0.3 to 1.0 mm, with the larger colonies predominating. The smaller, crowded colonies often were colorless, with bright yellow-to-yellow brown centers, whereas the more typical large colonies usually lacked such colorless borders.

After the initial incubation on the recovery broth, the membrane filters were transferred to the lysine-arginine agar and incubated for 1 h at 35°C in a GasPak anaerobic chamber (BBL). Longer incubation periods did not provide greater sensitivity and in fact resulted in false-positive reactions for crowded colonies. The plates were removed and incubated at room temperature for an additional 1 to 2 min to intensify the color reactions of positive colonies. Bright yellow-to-yellow orange colonies, as observed under fluorescent lighting with ×7 magnification, were marked by poking a hole with a sterile needle through the membrane next to each colony. Colonies of organisms exhibiting activity against either or both of the amino acids appeared blue, green, or greenish yellow.

The urease assay was performed immediately after the lysine-arginine assay by transferring the filter to an absorbent pad saturated with 2 ml of the urease broth. After a 5- to 10-min incubation period at room temperature, colonies of \textit{Y. enterocolitica} changed color from yellow to a distinct green or deep bluish purple, indicating urease activity. These colonies were often surrounded by a pink halo. Since the urease test gave a transient color reaction, it was essential to count the colonies promptly after the 5- to 10-min incubation period. Marked, green, or blue colonies represented those organisms which were sorbitol positive, lysine and arginine negative, and urease positive, respectively. These tests provided the basis for the presumptive identification of \textit{Y. enterocolitica}.

Results of specificity tests of the mYE technique, as determined by confirmatory identification tests of marked colonies recovered from suspensions of laboratory-prepared mixed cultures and natural water samples, are presented in Table 2. Verification of identity as \textit{Y. enterocolitica} was achieved for 88% of all colonies presumptively identified as such, whereas only 1.3% of the presumptive negative \textit{Y. enterocolitica} colonies were not confirmed as negative.

The accuracy of the mYE method was tested in several experiments. Quantitative recovery of pure-culture phosphate buffer suspensions, determined by comparison with recovery on blood agar spread plates, was demonstrated (Table 1) for the mYE recovery broth (91% recovery) and m-coliform broth (87% recovery). The range of recovery rates, however, was much larger for the m-coliform broth, and two strains exhibited

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of samples</th>
<th>Presumptive positive</th>
<th>Presumptive negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of colonies examined</td>
<td>No. confirmed positive (%)</td>
<td>No. of colonies examined</td>
</tr>
<tr>
<td>Laboratory-prepared mixed culture</td>
<td>7</td>
<td>171 (87.7)</td>
<td>226 (98.2)</td>
</tr>
<tr>
<td>Poudre River water</td>
<td>27</td>
<td>37 (89.2)</td>
<td>252 (99.2)</td>
</tr>
</tbody>
</table>
less than 50% recovery on this medium.

To evaluate the mYE technique under the more realistic conditions of low numbers of _Y. enterocolitica_ in a competitive environment, laboratory-prepared mixed cultures and samples of river water were seeded with low levels of the organism. A summary of the results is presented in Table 3. Fifty-four plates were examined, with an expected collective total of 520 CFU of _Y. enterocolitica_. The actual recovery was 470 CFU, or 90%. The background flora observed on the mYE plates ranged from 50 to 100 CFU per plate. Although it is acknowledged that the counts on these plates were below acceptable statistical standards, the results indicated that the actual mYE counts reflected the inocula fairly accurately, even at these low population levels in mixed culture. Identifications were confirmed for randomly selected positive colonies picked from the laboratory-prepared mixtures, and false identifications occurred at a rate of 2%.

To estimate the effective counting range for the mYE technique, river water samples were seeded with a variety of known concentrations of _Y. enterocolitica_. After equilibrating for 30 min at 10°C, the samples were plated and incubated, and the numbers of mYE-positive colonies were counted (Fig. 2). Recovery rates were high when the expected total counts (expected _Y. enterocolitica_ plus background organisms) on the filters ranged from 70 to 120 CFU per plate. Expected mYE counts (presumptive _Y. enterocolitica_) ranged from 10 to 65 CFU on these plates. However, as crowding of colonies on the plates increased, significant decreases in recovery were observed, as determined by a test of least significant difference at the 5% level.

**Field trials of the mYE technique.** Thirty-three cultures of _Y. enterocolitica_ were isolated during the field trials by using the mYE technique to recover this organism from natural aquatic sources. A summary of the biochemical characteristics of the isolates is presented in Table 4. All but one of the isolates were recovered from Poudre River water samples; the one exception came from prechlorinated sewage effluent. _Y. enterocolitica_ was isolated from 9 of 11 (81.8%) samples from upper river sites, located within the Roosevelt National Forest, and from 6 of 16 (38%) samples from lower river sites. Only five (15%) of the isolates were serotypable; two were O:4 (or O:4,32), two were O:17, and the fifth was O:40. Nine isolates (27%) fermented rhamnose, thus resembling the "environmental strains" designated by Brenner et al. (6) and originally described by Botzler et al. (5). Most of the isolates were classified as Nilehn biotype 1 or 4 (16).

**DISCUSSION**

The mYE technique is an improvement over the current methodology for the recovery of _Y. enterocolitica_ from water in the time required for isolation and identification, since a presumptive identification of the organism is obtained within 50 h. Previous schemes in which nonspecific media are used, such as MacConkey agar, salmonella-shigella agar, and m-coliform (m-Endo) broth, generally require a minimum of 48 h of incubation. However, these media provide information concerning only lactose fermentation, and many colonies must be selected and further differentiated, requiring additional time and media. Also, cold enrichment techniques call for incubation periods of several days or weeks, along with the use of additional plating and differential media.

The specificity of the mYE technique allows a substantial reduction in the number of colonies which must be screened to recognize the organism; 88% of the presumptive positive identifications in this study were confirmed (Table 2).

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**TABLE 3. Recovery of low levels of _Y. enterocolitica_ in mixed culture**

<table>
<thead>
<tr>
<th>Source of background organisms</th>
<th>No. of samples</th>
<th>mYE count per plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory-prepared mixed cultures</td>
<td></td>
<td>Expected&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Test run 1</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Test run 2</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>Poudre River water</td>
<td>6</td>
<td>11</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined by triplicate blood agar spread plates.  
<sup>b</sup> Each sample was tested with triplicate mYE plates. Each value represents the mean count of 18 plates.

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**FIG. 2. Estimation of the counting range for the recovery of _Y. enterocolitica_.** Symbols: ●, strain 8; △, strain 721618; ○, strain 79B1.
Y. enterocolitica was recovered with great regularity from the Poudre River. This finding is in agreement with the report of Saari and Quan (T. N. Saari and T. J. Quan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, C119, p. 45), which described the isolation of 100 strains of the organism from all major Colorado streams, including high mountain streams and also from some wells. Harvey et al. (9) have also reported the recovery of the organism from remote mountain areas in California. The source of Y. enterocolitica found in these high-quality surface waters and its public health significance are unknown. Despite the common occurrence of the organism in Colorado waters, there has never been a reported case of human infection acquired in the state. However, Wetzler and Mcclellan [T. F. Wetzler and D. Mcclellan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, C(H)69, p. 357] have reported the isolation of serotype O:4 (O:4,32) from patients suffering from gastroenteritis in the state of Washington. In addition, serotype O:17 has also been encountered in clinical cases (4). Both of these serotypes were isolated in this study. Y. enterocolitica may be of pathogenic significance in Colorado but remains unrecognized due to the special effort required, but rarely taken, to recover the organism from humans. Further work in this area is needed before this significance can be determined.

ACKNOWLEDGMENTS

The excellent technical assistance of Kirke L. Martin is gratefully acknowledged.

This study was supported in part by training grant T 900 266 from the U.S. Environmental Protection Agency.

LITERATURE CITED


8. Dudley, M. V., and E. B. Shotts. 1979. Medium for the


