Membrane Filter Procedure for Enumerating the Component Genera of the Coliform Group in Seawater

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A facile, quantitative, membrane filter procedure (mC) for defining the distribution of coliform populations in seawater according to the component genera was developed. The procedure, which utilizes a series of in situ substrate tests to obviate the picking of colonies for identification, also provides an estimate of the total coliform density. When pure cultures of Escherichia coli, Klebsiella pneumoniae, and Enterobacter cloacae were suspended in seawater and held at 4 C for 24 h, between 56 and 100% of the cells which grew on nutrient agar spread plates at 35 C could be recovered by the mC procedure. Confirmation as coliforms of typical colonies from natural samples was about 95%. Assay variability was found to be insignificant. The recovery of coliforms from marine waters by the mC procedure was comparable to those obtainable by current methods. Klebsiella was differentiated by the urease reaction and E. coli by its ability to form indole. The confirmation frequencies for colonies designated as Klebsiella and E. coli by the in situ tests approached 95% for the former and 98% for the latter.

Total coliform and fecal coliform densities have been used to indicate hazardous fecal contamination of surface as well as potable waters. The practice of using these two groups as indicators of the contamination of surface waters with fecal wastes or sewage has been questioned recently, particularly with regard to the health hazard related to the use of such waters (9). The ambiguity arising therefrom derives firstly from the fact that some coliform biotypes are widely distributed in nature and may be only transient inhabitants of the gastrointestinal tract of warm-blooded animals; secondly, the ability of some coliforms to simulate the behavior of microbial pathogens in the aquatic environment, especially in marine waters, appears to be inadequate (13, 21).

The shortcomings noted above are mainly due to the heterogeneity of the coliform group. The elevated temperature, fecal coliform test, either by the most-probable-number estimate or the membrane filter technique, was developed to alleviate these shortcomings. Although fecal coliforms are a more restricted portion of the total coliform population, they too are a heterogeneous group composed primarily of Escherichia coli and Klebsiella biotypes. More important, about one-half of the Klebsiella isolates obtained from certain industrial effluents free of fecal contamination would fit the definition of fecal coliforms (3).

Another approach toward increasing the specificity of coliforms as indicators has been to type them according to their IMViC reactions (indole, methyl red, Voges-Proskauer, and citrate tests) (20). However, IMViC typing presents some problems. Closs and Digranes (5) have shown that IMViC patterns are nonspecific relative to the genera of the coliform group. The ambiguity caused by IMViC typing is most evident with the genera Klebsiella and Enterobacter, both of which have the -- + + IMViC pattern. Klebsiella may be found in feces (18, 22), industrial wastes (3), or nature (8), whereas Enterobacter is most frequently found in extra-enteral environments (15). Second, IMViC typing requires the isolation and identification of individual colonies. Thus, to determine the meaningful relationships between sources of pollution and potential health hazards, laborious and time-consuming ancillary biochemical procedures are necessary. This necessity has probably held back many investigations along these lines. Geldreich (14) has stated: "Because of the number of sources contributing coliform bacteria to surface water, the more information that can be obtained as to those sources, the greater the value of the
organisms as indicators of pollution." Therefore, by implication, information about coliforms differentiated at the generic level would probably be of more value then past data collected on the basis of IMViC biotypes, if this could be accomplished in a simple and efficient manner.

This paper describes the evaluation of a method for quantifying coliforms according to their component genera, *Escherichia*, *Klebsiella*, and *Enterobacter-Citrobacter*. The method was developed specifically in response to the need for reexamining the distribution of coliform biotypes according to the nature and proximity of pollution sources.

**MATERIALS AND METHODS**

**Cultures.** The coliform strains used to test the accuracy of the method with pure cultures were isolated from water samples obtained from Narragansett Bay, R.I.

All the isolates were gram-negative, oxidase-negative rods which fermented lactose at 35°C with the production of acid and gas. The reactions on the indole, methyl red, Voges-Proskauer, Simmon's citrate, lysine and ornithine decarboxylase, arginine dihydrolase, urease, glucosante, hydrogen sulfide, potassium cyanide, and motility tests were typical for the species as described by Edwards and Ewing (10). The *Pseudomonas aeruginosa* and *Aeromonas hydrophila* strains are maintained as stock cultures at this laboratory and have been used in other studies. The *Serratia marcescens* and *Proteus mirabilis* cultures were obtained from C. Houston of the University of Rhode Island. The cultures were maintained on appropriate media during the course of this study. Bacteria used to test the sensitivity and selectivity of the medium were grown overnight in Trypticase soy broth incubated at 35°C. Then, they were serially diluted in phosphate-buffered saline (pH 7.4) to a predetermined density to yield 20 to 80 colonies/filter.

**Natural samples.** Field samples were collected from various marine waters in the northeast section of the United States. Water samples were put on ice immediately after collection and then transported to the laboratory as rapidly as possible. The coliform assays were performed within 6 h of collection.

**Control measures for accuracy determinations.** Recovery accuracy for pure cultures was measured relative to the number of colonies obtained on a nutrient agar spread plate. An appropriate dilution (0.2 ml) of the test suspensions was pipetted onto each of three nutrient agar plates. This volume then was spread evenly over the entire surface of the medium with a sterile glass rod. The plates were incubated at 35°C in an inverted position for 24 h.

Coliform recoveries from natural samples were measured against either the m-Endo or the total coliform most-probable-number method. These latter methods were performed as described in *Standard Methods for the Examination of Water and Wastewater* (1). The assumption was made that these methods would provide the best available estimate of the true coliform density in natural samples.

**Primary isolation.** (i) Filtration and counting. Appropriate volumes of the test suspensions or field samples were filtered through a 47-mm membrane filter having an average pore size of 0.45 μm. If the volume was less than 10 ml buffered saline was added to the filter funnel prior to delivery of the sample so that the final volume filtered was at least 20 ml. The membranes were applied to the surface of plates containing the coliform isolation medium (mC) which then were incubated in an inverted position at 35°C for 22 ± 2 h.

Colonies counted as coliforms were blue or green in color and usually greater than 0.2 mm in diameter; those considered as negative were gray or cream colored. Questionable colonies were examined further by observing the reverse side of the plate; those which exhibited a blue coloration at the location of the colony were recorded as positive.

(ii) Membrane filter, isolation medium. The selective-differential medium (mC) was obtained after extensive evaluation of nutrient sources and selective and differential agents. Initial attempts to produce a chemically defined medium were unsuccessful. Although various fermentable carbohydrates were tested, lactose still proved to be the most effective one for the differentiation of coliforms. Ammonium chloride was added as an available source of nitrogen. Glutamic acid and cysteine were added because they were shown by Gray (13) to increase the recovery of coliforms; proline also was found to optimize their recovery. Tryptophan was added to accelerate the induction of tryptophanase system in a subsequent in situ test for indole production. Twenty vitamins and growth factors were examined as substrates for yeast extract in increasing coliform recoveries. They were not effective either singly or in combination; therefore, yeast extract was retained in the medium. Numerous inhibitors of gram-positive bacteria were examined. Sodium taurocholate proved to be least inhibitory to the coliforms and most effective in reducing the indigenous gram-positive microorganisms. Vancomycin in the concentration given did not significantly reduce coliforms recoveries and did eliminate some of the background organisms. A dye intermediate, 2,4-dinitrophenol, which is yellow on the alkaline side of neutrality, was added to enhance the color contrast between lactose fermenters and nonlactose fermenters. In addition to this function it also acted as an inhibitor of noncoliforms. The mC medium was prepared as follows: L-glutamate (Calbiochem), 0.4 g; L-cysteine hydrochloride (Calbiochem), 0.05 g; L-proline (Calbiochem), 0.04 g; L-tryptophan (Calbiochem), 0.06 g; yeast extract (Difco), 0.2 g; ammonium chloride (Fisher), 0.2 g; lactose (Fisher), 0.8 g; salt solution, 50 ml (see below); and distilled water, 50 ml.

The pH was adjusted to 7.5 with 1 N NaOH, and 1.5 g of agar (Difco) was added. The ingredients were well mixed and then autoclaved at 121°C for 15 min. Before cooling, 1.0 ml of a 1% (wt/vol) aqueous solution of aniline blue (Matheson, Coleman and Bell) was added, and the mixture was allowed to
stand for 5 min. After it was cooled to 55 C, the following unsterilized constituents were added per 100 ml: sodium taurocholate (City Biochemical, New York, N.Y.), 50 mg; vancomycin (E. Lilly), 0.1 mg; 2,4-dinitrophenol (Calbiochem), 1 ml of a 0.1% (wt/vol) aqueous solution. The final pH of the medium was 7.1. The medium then was dispensed in 4-ml quantities into membrane filter plates (50 by 12 mm).

The salt solution was prepared as follows: NaCl, 0.2 g; KCl, 0.8 g; K$_2$HPO$_4$, 0.3 g; KH$_2$PO$_4$, 0.1 g; MgSO$_4$·7H$_2$O, 0.02 g; and distilled water, 100 ml.

(iii) In situ tests. These tests were designed so that the coliform colonies could be presumptively identified as to genus without subculturing them to media for biochemical testing. The tests were accomplished by sequentially transferring the filter to one or more substrate or end product indicator systems. The tests used were the (i) urease, (ii) oxidase, and (iii) indole tests in the order as given. The oxidase test generally is not required because oxidase-positive, lactose-fermenting organisms generally grow on this medium. All in situ tests were performed at room temperature (approximately 22 C).

(iv) Urease test. Each lactose-positive (blue) colony was located on the filter. This was done either by piercing the membrane with a syringe needle immediately adjacent to the positive colony or by photographing the plate with a polaroid camera and marking the positive colonies on the print for future reference. The filter was aseptically removed from the growth medium with flame forceps and placed on a pad saturated with urea substrate. After 15 to 20 min the urease-positive colonies were noted. They were identified by their magenta color. Nonblue colonies were frequently urease positive, but they could be ignored.

The urea substrate was prepared as follows: urea, 2 g; phenol red, 10 mg; and distilled water, 100 ml. The substrate solution was brought to a pH of 5.0 ± 0.2; it was a straw-yellow color at this pH. The substrate solution was stored at 6 to 8 C for no more than 1 week.

Urease-positive, lactose-positive colonies were marked a second time.

(v) Oxidase test. The in situ oxidase test was performed by the method of Daubner and Mayer (6). After completion of the urease test, the filter was transferred to a pad saturated with a freshly prepared 1% aqueous solution of N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride. The reaction (a deep purple color) developed within 10 s. The colonial integrity of each oxidase-positive, lactose-positive colony was obliterated with a wire loop so it would not be included in the coliform count; then the filter was transferred immediately to the tryptophan substrate for the indole test.

(vi) Indole test. An absorbent pad was saturated with the tryptophan substrate. The filter was then transferred from the area (in the event the oxidase test was not performed) or oxidase substrate pad to the tryptophan substrate pad. After the filter remained on the substrate for 20 min, it was transferred to another absorbent pad saturated with indole reagent. Indole-positive colonies, if present, were recognized within 10 min by their red color or red halo surrounding a blue colony. Many noncoliform colonies gave a positive indole test. However, only those colonies which were previously marked as coliforms (blue colonies) were counted.

The tryptophan substrate and indole reagents were prepared as follows. Tryptophanase substrate: 0.5 g of Trypticase and 0.2 g of tryptophan were dissolved in 100 ml of distilled water; then the substrate solution was adjusted to a pH of 7.2, sterilized by filtration, and refrigerated until used. Indole reagent: 5.0 g of para-dimethylaminobenzaldehyde was dissolved in 90 ml of ethyl alcohol; then 10 ml of concentrated hydrochloric acid was added to the solution.

(vii) Confirmation of colonies. Colonies from field samples, which were presumptively identified by the in situ tests as either Klebsiella pneumoniae or E. coli as well as some not identified as either of these genera, were confirmed by subculturing the colonies prior to the in situ indole test to nutrient agar and phenol red lactose broth. An oxidase test (19) was performed on the nutrient agar cultures, and the lactose tubes were examined for gas production. The lactose-positive, oxidase-negative isolates were subjected to further biochemical tests to determine the genera to which they belonged. The system proposed by Wolfe and Amsterdam (23) was used to accomplish this end. The media used in the confirmation of colonies and in the comparison of recoveries were m-Endo membrane filter broth, Simmon’s citrate agar, SIM medium, decarboxylase base (Moeller) with and without added ornithine as the substrate, eosin methylene blue agar, nutrient agar, and phenol red-lactose broth. These media were purchased in the dehydrated form from Difco.

RESULTS

The evaluation of the mC procedure considered a number of the characteristics of the method. These were accuracy, specificity, selectivity, precision, comparability to existing procedures, and differentiation.

Accuracy. The accuracy was established by comparing the ability of the medium to recover nonstressed and stressed coliforms relative to that of nutrient agar spread plates. The recovery of nonstressed organisms was examined from immediate assays performed on buffered saline dilutions of 18- to 20-h Trypticase soy broth cultures of the test organisms. The recoveries of 19 coliform strains from five genera were examined thereby; the results are shown in Table 1. A general description of the colonies for each genus is included in the table. The overall recovery rate of coliforms by the mC procedure relative to that on nutrient agar spread plates was approximately 95%. Relatively poor recoveries were obtained in only two instances, both with Enterobacter cloacae strains. In general, K. pneumoniae colonies
were about twice the size of those of the other members of the coliform group.

"Stressed" organisms were obtained by preparing the suspensions in filtered natural seawater and storing them at 4 C for 24 h. During this interval, the average survival as measured by the recoveries on nutrient agar spread plates was about 1% for *E. coli*, *K. pneumoniae*, and the two *Enterobacter* species. The average recovery on the mC medium relative to that on nutrient agar spread plates at 0, 3, 6, and 24 h for all strains was approximately 91% (Table 2). Although strain-to-strain differences were observed, they were not appreciable.

**Specificity.** The specificity of the medium was tested by examining samples of marine water from various locations in the northeastern section of the United States. Typical blue to blue-gray colonies designated as coliforms and those not fitting the coliform description were verified as to their identity using gas production in lactose broth at 35 C as the criterion. Where feasible, all of the colonies on a membrane filter plate were transferred to the verification medium. Table 3 shows the results of tests on more than 2,500 isolates. The overall confirmation rate for colonies designated as coliforms was 95%. Approximately 6% of the nonblue isolates were coliforms.

**Selectivity.** The selectivity of mC medium was determined by comparing the total recoveries (all colonies) from polluted marine water samples on mC medium to those on mC medium in which the inhibitors were omitted. The background microbial flora, which ranged in density from 10⁴ to 10⁹/ml, was reduced approximately 100-fold. In general, the 100-fold reduction in the background flora was sufficient for the enumeration of coliforms when the water sample examined (filtered) was 10 ml or less. Frequently, however, the examination of 100-ml samples was difficult or impossible due to overgrowth by background microorganisms.

**Precision.** The precision or reliability of the mC method, as applied to total coliform enumeration, was determined by the D² technique described by Eisenhart and Wilson (11). D² is defined by the equation: 

\[ D^2 = \frac{\left( \sum X_i^2 \right)}{\left( \sum X_i \right)^2} - \left( \frac{\sum X_i}{n} \right)^2 \]

where the plate counts \( X_1, X_2, \ldots, X_n \), and \( N \) (the

### Table 2. Recovery of seawater stressed coliforms² on mC medium

| Organism                | % Relative recovery² after |
|-------------------------|---------------------------|
|                         | 0 h   | 3 h   | 6 h   | 24 h  |
| *E. coli* (LS 2)²       | 100   | 57    | 67    | 69    |
| *E. coli* (LS 3)        | 133   | 72    | 113   | 86    |
| *E. coli* (LS 4)        | 77    | 97    | 100   | 100   |
| *K. pneumoniae* (LS 1) | 110   | 93    | 113   | 117   |
| *K. pneumoniae* (LS 2) | 83    | 99    | 79    | 54    |
| *E. cloacae* (LS 4a)    | 87    | 87    | 55    | 100   |
| *E. cloacae* (LS 9)     | 93    | 100   | 106   | 112   |

² Coliforms were stressed by suspending in filtered seawater and storing the suspensions for 24 h at 4 C.

³ Relative to that on nutrient agar spread plates at the designated hour.

⁴ Laboratory number is in parentheses.

### Table 3. Verification of colony types on mC medium

| Sampling location      | No. of samples | % of colonies which verify as coliforms |
|------------------------|----------------|---------------------------------------|
|                        | No. tested    | Typical (blue) | Atypical (nonblue) |
|                        | % Coliforms ² | % Coliforms ² | % Coliforms ² |
| Narragansett Bay       | 6             | 94            | 93            | 93            | 7.5              |
| Boston Harbor          | 5             | 256           | 91            | 154           | 9.7             |
| Connecticut Shore      | 3             | 229           | 100           | 133           | 1.5             |
| Wickford Harbor, R.I.  | 5             | 95            | 100           | 17            | 11.8            |
| New York Bay           | 256           | 1,496         | 93            |                |                 |

² Positive verification indicated by gas production in lactose broth.
number of replicate plates per sample) was 5. The $D^2$ values calculated from the examination of 24 polluted marine water samples, along with the expected $D^2$ control limits for $P = 0.005$, 0.025, and 0.5, are shown in Fig. 1. Observed $D^2$ values should be distributed evenly on either side of the $P = 0.5$ control limit line if the variability from sample to sample among the five replicate determinations (plates) is as expected. Furthermore, by chance alone, a value exceeding the $P = 0.025$ control limit is expected in at least one out of 40 determinations. As can be readily seen, the calculated $D^2$ values are evenly distributed around the $P = 0.5$ line with one exception.

**Comparability to existing methods.** The comparability of coliform recoveries by the mC procedure relative to those by Standard Methods for the Examination of Water and Wastewater techniques (1) was examined in 20 marine water samples collected from New York Bay (Table 4). The samples were assayed in parallel by the mC, m-Endo, and coliform most-probable-number methods. The recovery by the mC method was greater than that by the m-Endo procedure in 14 of 20 samples. Of the remaining six samples, the difference was less than 5% in one case. The mC procedure and the completed most-probable-number method were comparable in their coliform recoveries. The former recovered more bacteria than the latter in nine out of 20 samples, and the geometric means were almost identical.

**Table 4. Comparative recovery of coliforms by the mC, most-probable-number, and m-Endo procedures**

| Sample no.* | Verified coliform density/100 ml | mC* | m-Endo* | Most probable number (completed)* |
|-------------|---------------------------------|-----|---------|----------------------------------|
| 1           | 2,100                           | 3,400 | 1,100  |
| 2           | 4,100                           | 6,000 | 13,000 |
| 3           | 160                             | 84   | 490    |
| 4           | 1,200                           | 270  | 330    |
| 5           | 120                             | 250  | 490    |
| 6           | 170                             | 84   | 230    |
| 7           | 1,780                           | 1,080 | 3,500  |
| 8           | 3,170                           | 2,800 | 4,900  |
| 9           | 130                             | 136  | 78     |
| 10          | 600                             | 190  | 2,400  |
| 11          | 600                             | 330  | 1,300  |
| 12          | 700                             | 420  | 330    |
| 13          | 33                              | 0    | 0      |
| 14          | 10                              | 40   | 110    |
| 15          | 30                              | 4    | 17     |
| 16          | 110                             | 60   | 40     |
| 17          | 4                               | 10   | 0      |
| 18          | 30                              | 20   | 17     |
| 19          | 380                             | 300  | 170    |
| 20          | 260                             | 100  | 1,100  |

Geometric mean 219 137 222

*Ten samples from each of two sampling areas off Rockaways and Coney Island beaches, N.Y.

*Picked colonies are lactose gas positive.

*According to Standard Methods for the Examination of Water and Wastewater (1).

**Differentiation.** The total coliform density was obtained from the number of typical, oxidase-negative (when this test was done) colonies on mC medium. The *Klebsiella* component was obtained directly from the number of typical colonies which were urease positive using the in situ test. The *E. coli* component was obtained directly as the number of typical colonies which were indole positive and urease negative. The *Enterobacter-Citrobacter* component was estimated by subtracting the sum of the *Klebsiella* and *Escherichia* colonies from the total coliform count.

The validity of the in situ urease test for the presumptive identification of members of the genus *Klebsiella* was examined by verifying urease-positive and -negative colonies using the H₂S-ornithine decarboxylase-citrate system of Wolfe and Amsterdam (23). Of 190 urease-positive colonies examined, about 6% did not verify as *Klebsiella* (Table 5). False-negative reactions were observed in about 4% of 340 urease-negative colonies.
The in situ indole test was examined to determine its specificity in correctly identifying E. coli colonies. Of 239 urease-negative colonies which were picked for isolation and identification, 150 were E. coli, 84 were Enterobacter sp., and five were Citrobacter. One hundred and forty-five of the 239 isolates were positive by the in situ indole test (Table 6). Thus, about 2% did not verify as E. coli. Conversely, seven of the 94 (8%) in situ indole-negative colonies were subsequently identified as E. coli.

**DISCUSSION**

A necessary requirement for a medium designed to study bacterial distributions in marine waters is that it must accurately reflect their true density in a given environment. However, the true density of coliforms in a natural sample is almost impossible to determine. The estimate of the accuracy of the mC method, as examined in the present study, assumed that the density of the “stressed” test organisms as obtained from spread plates (membrane filter and pour plates purposely were not used) on a rich medium containing no inhibitors is a true reflection of the “recoverable” population. Second, it assumes that the stress as applied, 24 h in seawater, generally approximates the conditions that might be encountered in the environment. The mC medium was shown to accurately recover stressed and unstressed organisms of the genera Escherichia, Klebsiella, Enterobacter, and Citrobacter; therefore, in the context of the above assumptions, it should quantitatively recover these organisms from marine water samples. Other organisms which might be encountered in polluted marine waters (i.e., members of the genera Aeromonas, Pseudomonas, Serratia, Proteus, and group D streptococci) were tested under the same conditions and with one exception were found to be inhibited or differentiated. The exception, certain Aeromonas species, is usually reported as not being able to ferment lactose; however, lactose-positive biotypes produce colonies similar to those of the coliforms within the optimum incubation period. On the mC medium, these were encountered so rarely that the in situ oxidase test was omitted midway through the study. Nevertheless, these aeromonads, when present in sufficient number, can be differentiated from coliforms by an in situ oxidase test.

Another approach towards evaluating the accuracy of coliform estimates by the mC procedure, and one which can be used with environmental samples, is to compare the recoveries with existing “standard methods.” The results obtained indicate that the mC method is more efficient than the m-Endo technique and that it is approximately as efficient as the completed most-probable-number procedure for estimating coliform densities.

The precision or reliability of the mC method as demonstrated by the D² technique was very good. The plate-to-plate variability across samples was about that which would be expected by chance alone, indicating the absence of those factors which could lead to excessive or, even, too little variability. The one D² value which fell outside the P = 0.025 control limit was not unexpected; by chance alone one value in 40 should have done so.

The specificity of the total coliform estimates by the mC method was very good considering the heterogeneity of the coliform group. The overall false-positive rate was about 5%. Most of the false positives were labeled thusly because they did not produce gas from lactose at 35 C; a very small number were rejected either because they were oxidase positive or would not grow in the confirmatory medium.

The establishment of the accuracy, precision, and specificity of total coliform estimates as obtained from the mC procedure is important in and of itself. However, the findings are more significant in that they provide the basis for the primary function of the mC procedure, that of describing the distribution of and quantifying

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**Table 6. Verification of in situ indole test for identification of E. coli**

| Indole reaction | No. examined | % E. coli* |
|-----------------|--------------|------------|
| Positive        | 145          | 98         |
| Negative        | 94           | 8          |

* Defined as lactose (gas) and indole positive, and citrate and H₂S negative.
coliforms according to the component genera. The achievement of this objective also required an evaluation of the specificity of the in situ tests used for the differentiation.

The use of the urease reaction to differentiate *Klebsiella* from other coliforms has been used previously by Hormaeche and Munilla (16), Barry et al. (2), and Matsen (17). They were at least 93% successful in properly identifying *Klebsiella* through the use of a urea substrate, tube test performed at 50°C. The test proposed in this study utilizes a low pH which allows *Klebsiella* colonies to be distinguished from other coliform colonies while they are still on the filter. As a single criterion for distinguishing *Klebsiella*, the in situ urease test was very accurate, correctly identifying 95% of the isolates from the marine environment. Thus, the in situ urease test represents a simple, rapid, and reliable means of identifying *Klebsiella* colonies without the necessity of inoculating other media.

Delaney et al. (7) described an in situ indole test which, in conjunction with a carbohydrate-free medium incubated at an elevated temperature (44°C), was used for the identification of *E. coli*. Their results indicated an excellent correlation with the number of *E. coli* isolated by a low-temperature (35°C) nonselective technique. The Delaney technique has been modified for use with the MC medium. Since tryptophanase is repressed when a fermentable carbohydrate is present (4), it was necessary to place the filter on a carbohydrate-free tryptophan substrate broth to induce the indole-forming enzyme system prior to transferring the filter to a pad containing modified Erlich reagent. Of the coliforms, only *E. coli* and certain *Klebsiella* biotypes possess the enzyme tryptophanase. In the method used, *Klebsiella* colonies are identified prior to the indole test and marked so that the indole-positive *Klebsiella* will not be counted with the *E. coli*. Ninety-eight percent verification of the in situ indole-positive colonies as *E. coli* was considered quite satisfactory.

It is the limited selectivity of the MC medium which poses the only limitation to its use. The problem appears most acute in warm marine waters containing an abundance of oxidase-positive organisms. Overgrowth of the coliforms by the oxidase-positive organisms limits the sensitivity of the MC procedure when 100-ml quantities of such waters are examined. However, even under such conditions, the sensitivity of the method is satisfactory in those marine waters where recreational use and shellfish harvesting are of prime interest. A search for inhibitors of the background growth is continuing.

Although, as noted above, selectivity leaves something to be desired, the MC medium has nevertheless been shown to be an accurate, reliable method for isolating coliforms from the marine environment. Furthermore, its compatibility with in situ tests for the presence of urease and tryptophanase allows for a rapid, presumptive identification of *E. coli*, *Klebsiella*, and *Enterobacter-Citrobacter* colonies without recourse to laborious ancillary identification measures.

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