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Membrane Filter Method for Enumerating *Escherichia coli*

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A membrane filter procedure for enumerating *Escherichia coli* was developed and evaluated. The method quantifies *E. coli* within 24 h without requiring subculture and identification of isolates. It incorporates a primary selective-differential medium for gram-negative, lactose-fermenting bacteria; resuscitation of weakened organisms by incubation for 2 h at 35°C before incubation at 44.5°C for 18 to 22 h; and an in situ urease test to differentiate *E. coli* from other thermotolerant, lactose-positive organisms. The recovery of *E. coli* from marine, estuarine, and freshwater samples exceeded 90%. Of the presumptively positive colonies, 91% were verified as *E. coli*. Less than 1% of all of the verified *E. coli* colonies failed to react typically.

The pioneering work of Snow and Budd in 1855 relating enteric disease to water contaminated with fecal wastes and the subsequent identification of the causal agents in the early 1880's (16) prompted investigators to search for a means of indexing water quality. In 1885, Escherich (9) observed that *Bacillus coli* (*Escherichia coli*) not only occurred in high densities in feces but also was frequently associated with the typhoid bacillus and, therefore, that it might be used as an indicator of fecal contamination. Several years later, Eijkman (8), working on a method to detect *E. coli*, found that gas formation in glucose broth incubated at 46°C was a rapid means by which to distinguish this organism. In 1929, Leiter (13) reported that the combination of the Eijkman test and the test for indole production was almost specific for *E. coli*. Since that time, many investigators have devised tests to enumerate *E. coli* based on the ability of this organism to produce indole from tryptophane at an elevated temperature (6, 14, 15, 20). The use of the indole test to identify *E. coli*, however, has two inherent disadvantages. First, the determination of indole production in conjunction with a most probable number procedure usually requires the transfer of bacteria from lactose broth to another medium and an additional 24-h incubation period. Second, the reagents used to perform the indole test are bactericidal, and, therefore, further examination of the colonies under consideration is impossible.

The purpose of this study was to develop a simple, accurate, nonlethal membrane filter technique for the rapid enumeration of *E. coli*.

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MATERIALS AND METHODS

The medium formulated for the enumeration of *E. coli* (mTEC) contains ingredients common to a number of coliform media. It has the following composition: Proteose peptone no. 3 (Difco), 5.0 g; yeast extract (Difco), 3.0 g; lactose (Fisher Scientific), 10.0 g; NaCl, 7.5 g; K_2HPO_4, 3.3 g; KH_2PO_4, 1 g; sodium lauryl sulfate (Matheson, Coleman and Bell), 0.2 g; sodium desoxycholate (Fisher Scientific), 0.1 g; bromocresol purple (Nutritional Biochemical Corp.), 80 mg; bromophenol red (Matheson, Coleman and Bell), 80 mg; agar (Difco), 15 g; and distilled water, 1 liter. The ingredients are dissolved by stirring, sterilized by autoclaving at 121°C for 15 min, and poured into plates (10 by 50 mm; 4 ml per plate). The pH of the medium was 7.3 ± 0.1.

Water samples were collected at freshwater, estuarine, and coastal water sites in Connecticut, Massachusetts, New York, and Rhode Island. Secondary treated effluents were obtained from local Rhode Island sewage treatment plants. All samples were assayed within 3 h after collection, with the exception of those samples from New York, which were assayed within 6 h.

Appropriate volumes of each sample were passed through 0.45-μm porosity membrane filters (Gelman), and the filters then were placed on the medium. The plates were then incubated in a 35°C incubator for 2 h. After this resuscitation period, the plates were put into watertight plastic bags which were then immersed in a 44.5°C water bath. After 20 ± 2 h, the plates were removed from the water bath, and the countable filters were transferred to pads saturated with urea substrate for an in situ urease test (7). Fifteen minutes after placing the filters on the urea substrate, all yellow colonies were counted and recorded.

The optimum resuscitation time was determined by using natural samples that had been filtered in the usual manner, held for various periods up to 4 h at 35°C, and then incubated at 44.5°C for approximately 20 h. The results from the three replicate plates at

each time interval were then compared with those that had been directly placed in the 44.5°C water bath.

The performance of the method was evaluated for specificity, accuracy, precision, and counting range.

Specificity. The specificity of the mTEC procedure was evaluated as follows. After the in situ urease test, yellow colonies were verified as *E. coli* by subculturing each colony to nutrient agar (Difco) and Simmons citrate plates which were incubated at 35°C. Twenty-four hours after inoculation, the nutrient agar cultures were tested for oxidase (18) and urease (2) activities, and the citrate plates were observed for growth. Yellow colonies which were oxidase, urease, and citrate negative were considered to be *E. coli*. Non-yellow colonies observed before the in situ urease test were subcultured to lauryl sulfate-lactose broth (Difco). Gas-positive tubes were carried through the same scheme used for the target colonies. (see above). Colonies that were lactose and gas positive and oxidase, urease, and citrate negative were designated as *E. coli*. The percentages of false-positive and false-negative colonies were determined relative to the total number of target colonies examined and the total number of verified *E. coli* colonies, respectively.

Accuracy. Accuracy was judged on the basis of two different procedures.

The first procedure involved the use of artificially stressed pure cultures of environmental isolates of *E. coli* that were enumerated on noninhibitory media and by the mTEC method. Details of this procedure are described elsewhere (7).

The second, and more realistic, procedure compared the recovery of *E. coli* in natural samples by the mTEC procedure with that obtained with a nutrient-rich, noninhibitory reference medium. Plate count agar (PCA) (Difco) was initially used as the reference medium for determining the expected number of *E. coli*, but it was rejected after finding that this medium was frequently overwhelmed by gram-positive bacteria in natural samples. Levine eosin methylene blue agar (EMB) (Difco) was chosen as a substitute after it was determined that the recovery of numerous *E. coli* strains of environmental origin was the same as that found with PCA. An estimate of the "true" *E. coli* density in each water sample was obtained by making appropriate dilutions in phosphate-buffered saline (7) and pipetting 0.2 ml of each dilution onto each of five EMB plates. The 0.2-ml amount was then spread over the entire surface of the medium with a sterile glass rod. The EMB plates were incubated in an inverted position in a 35°C incubator for 24 h.

The *E. coli* density was obtained by selecting a five-plate series whose collective sheen or nucleated colony count was in the 20 to 80 range. At least 20 of these colonies from each sample were transferred to lauryl sulfate-lactose broth. Gas-positive cultures were identified as *E. coli* by subjecting them to the verification tests described above. Verified *E. coli* cells produced gas from lactose at 35°C and were negative for the citrate utilization, urease, and oxidase tests. The verified *E. coli* count was used to extrapolate to the true *E. coli* density in each sample.

With both of the above procedures, accuracy was determined as the portion of the true density (as enumerated by the reference medium) obtained by the mTEC method.

Precision. The precision of the mTEC procedure counts was evaluated in terms of the standard deviation of the mean. The error was expressed as a percentage of the mean by using a formula given by Niemela (17):

$$\text{percent error} = \frac{s/\sqrt{n}\ (100)}{\bar{x}} \quad (1)$$

where s is the standard deviation, n is the number of plates examined, and $\bar{x}$ is the mean number of colonies per filter. The 95% confidence limits of the mean were calculated by multiplying the percent error by 2 (1). The mean number of colonies per plate and the standard deviation were calculated by the formulas given by Stearman (22):

$$\bar{x} = \frac{\sum x}{n} \quad (2)$$

$$s = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}} \quad (3)$$

where x is the number of colonies per plate and n is the number of plates.

The 95% confidence limits of the theoretical percent error of the mean of a population of plate counts were calculated from the following equation:

$$\text{theoretical percent error} = \frac{200}{\sqrt{n\mu}} \quad (4)$$

where n is a hypothetical number of plates and μ is a hypothetical population mean. The above calculations assume that bacterial counts follow a Poisson distribution (22) and, therefore, that the mean and variance are equal. The Fisher index of dispersion test (10) was used to determine if the replicate counts from each sample followed a Poisson series.

Upper counting limit. The limit beyond which the accuracy of the method degenerates due to overcrowding of target organisms or interspecies inhibition was determined by using multiple dilutions of natural samples filtered through five replicate filters. Samples were chosen so that they contained no less than 100 target organisms per 100 ml of the undiluted sample and generally no more than 2,000 per 100 ml. Dilutions were made such that the number of colonies decreased in increments of about 10% of those in the undiluted sample per filter. The final volume filtered equaled that of the undiluted sample. Before making dilutions, a large volume of sample was passed through a membrane filter having a 0.45-μm pore size; the resulting filtrate was used as the diluent. For each dilution series, an arbitrary reference count, the count nearest 30, was chosen as a compromise between a small number error and those errors noted above. Since it was assumed that the counts per filter would be a linear function of the dilution factor, each expected count in the series was obtained by multiplying the reciprocal of its dilution factor by the reference point factor (obtained by dividing the reference count by the reciprocal of its dilution factor).

The expected and observed values were examined with the statistical significance test given by Hald (12) to determine if the magnitude of the differences was

great enough to indicate that something in addition to random processes was responsible.

RESULTS

The effect of the 35°C preincubation period on the resuscitation of injured or debilitated bacterial cells is shown in Fig. 1. During the first 60 min, there was an increase of 66% in the *E. coli* recovery rate. At between 1 and 2 h, there was an increase of 20% of the average count at 1 h. Although the recovery rate was higher at 4 h than at 2 h, the latter resuscitation period was used throughout the study because of the greater logistic convenience of the shorter period.

The effect on resuscitation of preincubation at ambient temperatures, as opposed to 35°C, was also examined, and these results are shown in Fig. 2. There is a balanced distribution of points about the line of equality, indicating that these two resuscitation treatments were probably not different in their effect on *E. coli* recoveries. However, the absolute differences between the recovery values were greater with the 35°C resuscitation treatment, and, therefore, this temperature was used throughout the study.

The specificity of the mTEC method for recovering *E. coli* in various types of surface waters and sewage effluents is shown in Table 1. Colonies presumed to be *E. coli* (yellow colonies obtained after the in situ urease test) were confirmed with the oxidase, urease, and citrate utilization tests. Of the urease-negative, yellow colonies, 91% were verified as *E. coli*. False-positive colonies occurred more frequently in freshwater than in marine waters, with the respective rates being 15 and 6%. Less than 1% of the colonies which did not meet the above criteria were verified as *E. coli*. Background colonies averaged about 15% of the total count per plate; the range was 3 to 80%, depending on the source of the sample.

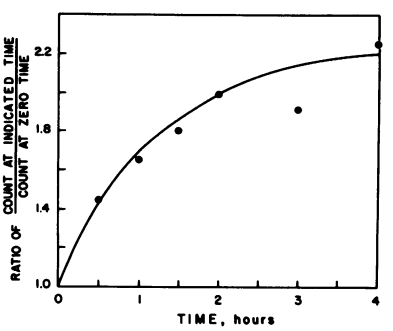

FIG. 1. *Effect of 35°C preincubation time on the recovery of E. coli from natural water samples.*

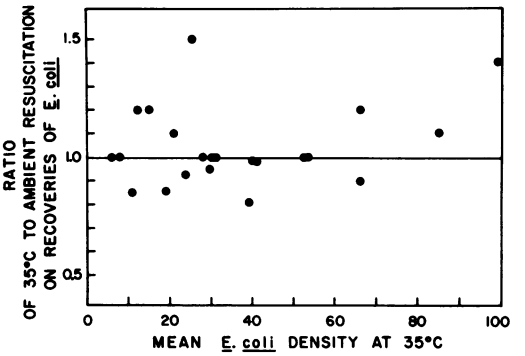

FIG. 2. *Comparison of 35°C and ambient temperature preincubations for resuscitating E. coli sampled from natural water environments.*

TABLE 1. *Verification of target colonies as E. coli*

Sample source	No. of target colonies	No. verified as *E. coli*	% verified as *E. coli*
Freshwater	263	223	85
Estuarine water	280	272	97
Coastal water	90	85	94
Sewage treatment plant effluent	208	181	87

The relative recoveries of *E. coli* from suspensions that had been stressed for 24 h at 11°C in nutrient-deficient filter-sterilized surface water are shown in Table 2. The overall relative recovery rate in seawater was higher than that in freshwater. The mean recovery rate for all of the *E. coli* strains in both fresh and marine waters was 98%, using PCA as the reference medium. Similar recovery rates were obtained when EMB was used as the reference medium. The relative recoveries of *E. coli* from natural samples, using Levine EMB agar as the reference medium, are shown in Table 3. The recovery of *E. coli* with the mTEC procedure was comparable to EMB medium in estuarine and coastal waters. In the freshwater samples, however, the detection rate was two to eight times higher with the mTEC method than with the EMB medium. The reason for this discrepancy was not obvious since there were no apparent differences with pure cultures inoculated into freshwater and seawater.

As the first step in characterizing the precision of the mTEC method, each three-plate replicate series was tested to determine if the data were randomly distributed and, therefore, if they could be treated with Poisson statistics. Those replicates for which the variance/mean ratio was higher or lower than that expected from a random distribution were not used for precision

TABLE 2. *Relative mTEC recoveries of artifically stressed[a] E. coli*

E. coli strain	*E. coli* recoveries (% of reference medium)[b]			
	Freshwater		Seawater	
	PCA	EMB	PCA	EMB
EC5	80	83	93	83
EC496	87	86	80	94
EC497	97	97	121	103
EC498	81	98	118	152
EC499	138	102	100	94
ECIP	84	87	82	112

[a] Cells were washed once with filtered freshwater or seawater diluent, resuspended in 10 volumes of the respective diluent, and held for 24 h at 11°C.

[b] $\frac{\text{Colonies on mTEC medium}}{\text{Colonies on PCA or EMB medium}} \times 100$

calculations. The 95% confidence limits of the standard deviation of the mean, expressed as a percentage of the mean, were calculated (equation 1) for all three-plate replicates whose counts were randomly distributed. Only 2 of 82 values were rejected on that bases. The remaining 80 error estimates were compared with a calculated (equation 4) hypothetical three-plate random sampling error curve (Fig. 3). The curve represents the limiting precision of the method, since replicate counts conforming to a Poisson series can never be less variable than that due to random sampling alone. In addition to random sampling error, all assay procedures have technical error. These two types of error contribute to the total variation represented by each data point (Fig. 3). If the conventional lower counting limit of 20 colonies is accepted, then only 5.8% of the means had error terms of greater than ±30%, where the means ranged from 20 to 160 colonies per filter. Of all the means, 35% had error terms greater than that expected from random sampling error alone.

The range over which colonies can be accurately counted is shown in Fig. 4. An appreciable displacement of the coordinate points from the line of equality is not observed until high values are encountered. A total of 10 values deviated from unity by differences greater than expected by chance alone. The observed count at which statistically significant differences began to appear was 83 colonies per plate.

DISCUSSION

Childs and Allen (5) in 1953 demonstrated the resuscitating effect of a 1-h preincubation at 35°C for coliform bacteria that were subsequently incubated at 44°C. Pretorius (19), Burman (4), and Rose et al. (21) used a 2-h resuscitation period at 37°C. Green et al. (11) found optimum recoveries of chlorine-stressed coliform bacteria at 5 h. In the present study, the 4-h resuscitation time at 37°C gave the highest average recovery rates. If the 4-h recoveries are considered as 100%, then the 2-h resuscitation period results in a rate that is only 12.5% lower. The logistic advantages of the 2-h period outweighed the small loss that occurred with the shorter resuscitation time relative to the 4-h period, and for that reason, the shorter preincubation period was adopted. None of the reports cited above (4, 5, 11, 19, 21) examined the utility of an ambient temperature resuscitation. A comparison between ambient temperature (24°C) and 35°C resuscitations (2 h) in this study indicated that, with natural samples, an appreciable difference was not observed between these two preincubation treatments. Thus, either technique should be acceptable. However, the mean recoveries at 35°C were higher than those at ambient temperature, and, therefore, the 35°C resuscitation temperature is recommended.

The specificity of the mTEC method appeared to be consistently greater in estuarine and coastal marine waters than in freshwater and sewage effluents. The higher frequency of false-positive colonies in freshwater locations could not be accounted for on the basis of different polluting sources since all of the sampling locations were in the vicinity of sewage treatment plants and should have received similar coliform distributions. It is possible, however, that the

TABLE 3. *Comparison of verified E. coli recovery rates with EMB agar medium and the mTEC method*

Sample source	No. of *E. coli* recoveries/ml		mTEC/EMB ratio
	EMB (verified)	mTEC (verified)	
Freshwater	51	102	2.0
	10	60[a]	6.0
	6	49[a]	8.2
	6	12	2.0
Estuarine water	20	18	0.90
	60	52	0.87
	40	38	0.95
	10	28[a]	2.8
Coastal water	70	85	1.2

[a] The magnitude of the observed differences between the reference and test method densities would occur less than 1 time in 20 in a pure randomization experiment.

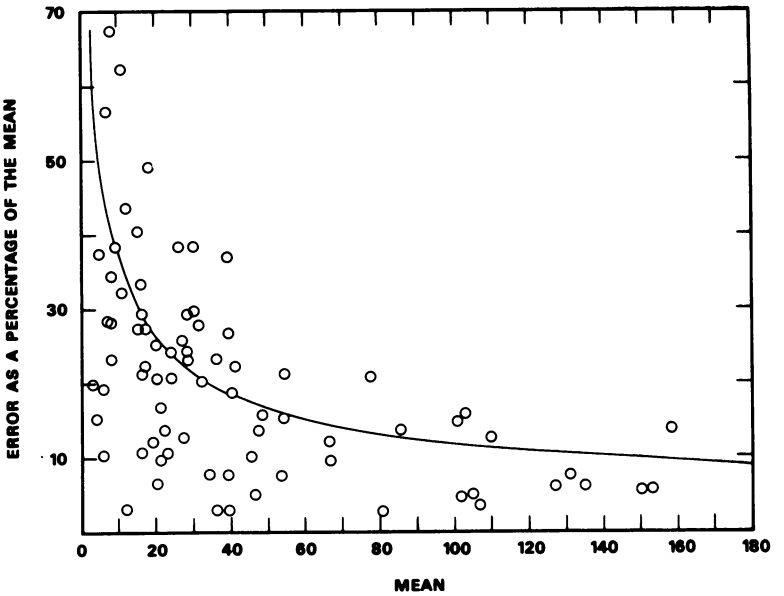

FIG. 3. *Theoretical (solid line) and observed* (○) *relative precisions of mean E. coli densities obtained with the mTEC method.*

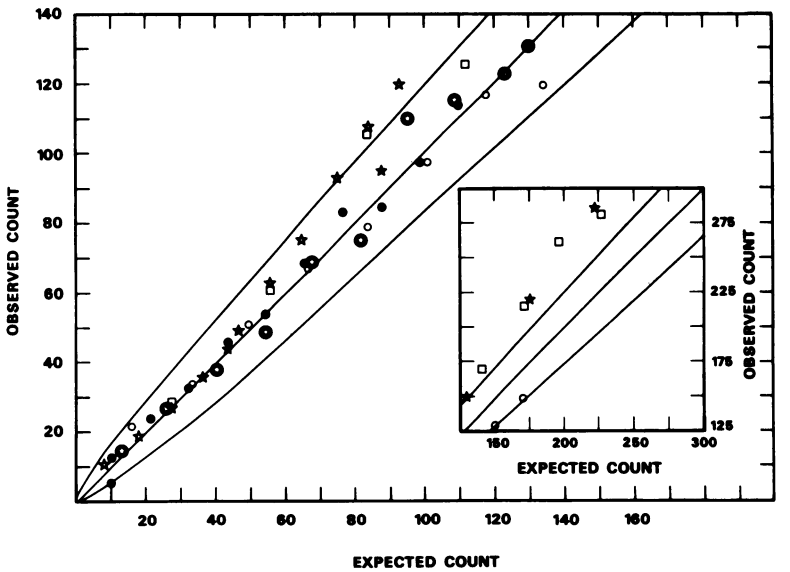

FIG. 4. *Comparison of expected and observed recoveries of E. coli from natural water samples. Lines on either side of the line of equality indicate 95% confidence limits for the expected counts (12). Each symbol represents a different water sample.*

thermotolerant and urease- and citrate-positive portion of the coliform distribution capable of growth on the medium has a greater capacity to survive in freshwater situations. Lactose- and oxidase-positive colonies were not recovered in any of the samples. Although it is evident that the nonspecificity occurs most often with freshwater samples, the frequency of false-positive colonies was not high enough to preclude its use in those environments.

Most media designed to select for a specific bacterial species generally use one or more inhibitors, employ restrictive temperatures, or contain limited nutrients. All of these factors,

which are used to suppress growth of unwanted background flora, may also inhibit some of the bacteria that the media are supposed to enumerate. If a portion of the target population is systematically not recovered from sample to sample, then this constant error becomes a characteristic of the method. Nevertheless, the accuracy of the mTEC method, whether examined with stressed cultures or natural samples, was comparable to those of the reference media. The higher recoveries from freshwater samples by mTEC relative to EMB may have been due to some factor or factors in fresh, sewage-polluted waters which interacted with a constituent of EMB medium, perhaps eosin or methylene blue, to inhibit *E. coli*. The estuarine water sample which was similarly affected was taken from a point below the mouth of a river that receives considerable sewage effluent.

The precision of any microbiological counting method is affected by two sources of error, sampling error and manipulation error (23). Manipulation error can usually be minimized by giving careful attention to the details of the method. Sampling error, however, can be minimized only by increasing the number of replicates. In most cases, this is logistically impractical, and some uncertainty about the mean is accepted. The precision of data presented in this study was for three replicate plates from each sample, and it is fairly representative of what would be expected from Poisson-distributed counts. The error terms follow the expected curve for three replicate plates rather well. The majority of those values that fall above the expected curve can be assumed to have resulted from manipulation error.

The upper counting limit of colonies on plates or filters is usually influenced by overlapping, where two colonies are counted as one, or antibiosis, which is usually a function of overcrowding by other species. The usual result of either of the above is an underestimate of the true density. This was not the case with the mTEC method. At high counts, the departures from the expected were always overestimated. These were due to urease-positive colonies which failed to give a positive in situ urease test because of their proximity to several acid-producing *E. coli* (urease-negative) colonies. However, if the counts do not exceed 80 colonies per filter, overestimates of the *E. coli* densities are unlikely to occur.

The mTEC method presented in this study differs radically from other methods published previously. In the past, membrane filter enumeration of *E. coli* was based on its ability to catabolize tryptophane to indole (6). Indole-positive colonies were detected by placing the filter on a pad saturated with Ehrlich reagent. The mTEC method, however, assumes that the majority of the thermotolerant coliforms that are not *E. coli* are urease positive and can be easily differentiated. The specificity data obtained in this study would indicate that this assumption is a valid one. There are two advantages to using this approach. The first is that lactose can be used as the carbohydrate source. Lactose can only be used with methods that rely on the production of indole if tryptophanase is subsequently induced on a non-lactose-containing medium (3, 7). The second advantage is that the in situ urease test, unlike the indole test, is not lethal to colonies. In the latter, the concentrated sulfuric acid in Erhlich reagent kills the organisms and prevents further testing.

The mTEC method appears to be quite suitable for the enumeration of *E. coli* in freshwater, estuarine water, and marine water. Of the colonies that were specified as *E. coli*, 91% were confirmed, and less than 1% of all of the verified *E. coli* colonies were identified as false-negative colonies. The accuracy of the method was well above 90%; however, it is affected by overestimates at high counts. Therefore, the use of plates containing more than 80 colonies is not recommended. The consequence of this limitation is that if precision is to be maintained at low densities, the number of replicate filters at each dilution will have to be increased, or some other means of increasing the number of colonies per filter will be required. Lastly, it should be pointed out that the data presented in this study were obtained from samples collected only in the northeastern section of the United States. Therefore, it is recommended that before its use elsewhere, a minimal number of samples should be examined locally to determine whether the specificity, accuracy, and precision characteristics are affected by local environments.

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