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Comparison of Membrane Filter, Multiple-Fermentation-Tube, and Presence-Absence Techniques for Detecting Total Coliforms in Small Community Water Systems

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Methods for detecting total coliform bacteria in drinking water were compared using 1,483 different drinking water samples from 15 small community water systems in Vermont and New Hampshire. The methods included the membrane filter (MF) technique, a 10-tube fermentation tube (FT) technique, and the presence-absence (P-A) test. Each technique was evaluated using a 100-ml drinking water sample. Of the 1,483 samples tested, 336 (23%) contained coliforms as indicated by either one, two, or all three techniques. The FT detected 82%, the P-A detected 88%, and the MF detected 64% of these positives. All techniques simultaneously detected 55% of the positives. Evaluation of the confirmation efficiency of the P-A technique showed 94% of the presumptive positives confirming as coliforms. Thirteen different species of coliforms were identified from the 37 tests in which the P-A was positive but the MF and FT were negative. The P-A test was simple to inoculate and interpret and was considerably more sensitive than the MF and slightly more sensitive than the FT in detecting coliforms in this type of drinking water supply.

Currently, the membrane filter (MF) technique and the multiple-fermentation-tube (FT) technique, as described in *Standard Methods for the Examination of Water and Wastewater* (1), are the only procedures approved for monitoring drinking water systems for total coliforms under the Safe Drinking Water Act (14). Both techniques have been evaluated and compared in several studies (4, 13, 19, 20, 22, 24). The conclusions from these studies have suggested modifications of the media and procedures of both methods for more accurate results (11, 13, 17, 18, 22). Other investigators have proposed a presence-absence (P-A) technique as an alternative (7, 8, 23, 25).

The P-A technique, a basic simplification of the FT procedure, was developed by J. A. Clark as a qualitative means of monitoring drinking water systems. Although it was tested in parallel with the MF on different drinking water systems and found to be as sensitive (7), it has not been compared with both the FT and MF together.

In this study, the sensitivity of each of the methods (MF, FT, and P-A) was compared using drinking water samples from a variety of small community water systems. The methods used were the conventional MF technique, a 10-tube FT technique, and the most recently proposed P-A technique (1). Small water systems located in rural areas are quite variable and are often less well protected than municipal supplies. In addition, appropriate laboratory facilities for monitoring are not readily available. The results presented in this study should assist in developing appropriate methods for improving this type of water supply.

MATERIALS AND METHODS

Samples. Over a 1-year period, a total of 15 small community water systems in Vermont and New Hampshire were sampled. These water systems (Table 1) each serve less than 1,000 persons and have more than 10 service connections. Sources of water for these systems include shallow wells, deep wells, and springs. Most systems did not chlorinate or filter their water. Samples were collected from one location in each system on a weekly basis. Once a month, five samples were collected from five sites in the distribution network of each system, including the weekly site.

Samples were collected according to the guidelines in *Handbook for Evaluating Water Bacteriological Laboratories* (16). Sterile 500-ml plastic bottles (polymethylpentene) containing sodium thiosulfate (16) were used as sample containers. Samples were kept in an ice chest, transported to the laboratory within 2 to 3 h, and analyzed within 5 to 6 h after collection.

Microbiological procedures. Each sample was analyzed by each of the techniques, using 100-ml water portions for each test. The MF procedure was performed by methods detailed in references 1 and 5. HA membrane filters (Millipore Corp., Bedford, Mass.) were placed on sterile pads (Millipore Corp.) saturated with M-endo broth (Millipore Corp.; Difco Laboratories, Detroit, Mich.) and incubated for 24 h at 35°C. A minimum of five of the typical green-metallic sheen colonies from each positive sample were transferred to lauryl tryptose broth (LTB; Difco) and brilliant green bile lactose broth (BGLB; Difco) for verification as coliforms (5). Production of gas in LTB and BGLB within 48 h was considered a positive test (5). At least one colony per positive sample was examined for lactose fermentation after growth on Levine eosin methylene blue (EMB) agar (Difco).

The FT technique involved 10 tubes, each containing 10 ml of double-strength LTB and a fermentation tube. The addition of a 10-ml sample of water to each tube allowed a total of 100 ml of water to be examined. For each positive sample, all presumptive positive tubes up to a maximum of

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TABLE 1. Description of the 15 drinking water systems

System no.	Year established	No. of service connections	Approx. no. of users	Water source[a]	Chlorination[b]	Filtration[c]
011	1928	11	30	1	−	−
021	1930	70	240	1	−	−
031	1960	20	55	1/5	+	−
041	1946	238	910	1	+	−
051	1965	38	90	2/5	+	−
061	1960	17	70	3	−	−
071	1965	18	55	1/5	−	−
081	1900	250	650	1	+	−
091	1949	300	930	1/5	−	−
1011	1977	40	75	1	+	+
1021	1968	30	65	1/5	−	−
1031	1952	13	30	1/5	−	−
1041	1935	70	325	2	+	−
1051	1970	98	350	2	+	+
1061	1948	135	420	1/5	−	+

[a] Water source: 1, deep well; 2, shallow well; 3, springs; 4, wells to reservoirs; 5, wells to covered reservoirs.
[b] Chlorination: −, none; +, chlorination with hypochlorite.
[c] Filtration: −, none; +, yes.

five were confirmed for gas production in BGLB. As indicated in Results, these water samples demonstrated a high confirmation efficiency, and therefore the confirmation of five tubes was sufficient to judge a given sample as contaminated with confirmed coliforms. At least one BGLB tube was transferred to Levine EMB agar. The number of coliforms per 100 ml was estimated from a 10-tube most-probable-number (MPN) table (1). The procedure was the same as previously described (5), with the omission of the final step of the completed test, in which typical colonies on EMB agar are transferred to LTB. To assess the validity of this modified procedure, a resampling scheme was conducted in which 49 positive samples (14% of the total number of positive samples of this study) were carried through to the final step of the completed test (see results below).

The P-A technique used was a recent modification of the original test proposed by Clark (7). The test consisted of a single culture bottle (250-ml milk dilution bottle), containing a 50-ml portion of triple-strength medium plus a fermentation tube (12 by 75 mm). The formulation of the medium at single strength was: 13.0 g of lactose broth (Difco), 17.5 g of LTB, and 0.0085 g of bromocresol purple (Difco) (dissolved in 10 ml of 0.1 N NaOH before addition to broth) in 1 liter of distilled water. The prepared bottle was autoclaved for 12 min at 121°C and was stored until used. Before samples were added, the bottle was inverted to empty the medium out of the fermentation tube. After the bottle had been inoculated with a 100-ml sample portion, it was inverted to fill the fermentation tube. The bottle was incubated at 35 ± 0.5°C and inspected after 24 and 48 h for production of acid or acid plus gas. Bottles showing any degree of color change from purple to yellow or brownish yellow were subcultured for confirmation. Inoculum from a presumptive positive test was transferred to BGLB for confirmation and then to Levine EMB agar for detection of lactose-fermenting colonies.

In cases in which the P-A test was positive and the MPN (FT) and MF methods were negative, organisms were isolated in pure culture from Levine EMB agar and identified using API 20E identification test strips (Analytab Products, Inc., Plainview, N.Y.). The species names given are those obtained from the API identification scheme.

A quality assurance program was performed as outlined in reference 5.

Statistical methods. McNemar's test (15) was used to compare the overall proportion of positive samples detected by different methods. This test does not take into account the disagreement on individual samples. To study patterns of agreement between methods, kappa measures of inter-rater agreement (2, 15) were used. Kappa is an index taking values between −1 and 1, indicating the relative agreement between two techniques beyond chance agreement. $\kappa = 1$ indicates maximum possible agreement; values greater than 0.75 represent excellent agreement, and values below 0.4 represent poor agreement beyond chance. $\kappa = 0$ indicates chance agreement only.

A further analysis was performed to study where disagreements of the P-A and the FT with MF occurred. This analysis was based on the obviously unproven assumption that MF gives the correct conclusions. The MF technique was chosen as the standard for comparison because of its wide usage among water laboratories. Conditional kappa measures of agreement (2) with FT were calculated given MF counts of 0, 1 to 4 (inclusive), and ≥5 per 100 ml. By collapsing the 1 to 4 (inclusive) and ≥5 categories into a ≥1 category, an additional measure of agreement was computed conditional on a positive MF count. Due to the dichotomous nature of the P-A test, conditional kappa measures could only be computed given zero and positive MF counts.

RESULTS

Comparison of the MF, FT, and P-A methods. Of the 1,483 samples analyzed in this study, 336 (23%) confirmed positive samples were detected by either one, two, or all three of the techniques. The total coliform counts of these samples ranged from 1 to over 300 organisms per 100 ml. A comparison of the three methods in detecting the presence of coliforms is shown in Fig. 1. The FT technique detected 275 positive samples (19% of the total samples, 82% of all positives), whereas the MF technique detected 216 positive samples (15% of the total samples, 64% of all positives) and the P-A technique detected 296 positive samples (20% of the total samples, 88% of all positives). All three techniques simultaneously detected coliforms in 185 samples. There was a statistically significant difference, when analyzed by McNemar's test (15), in the detection rate of positive samples between each pair of methods. The MF detected fewer

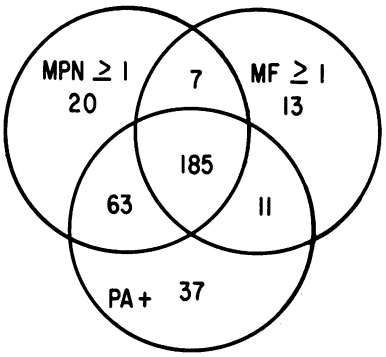

FIG. 1. Comparison of three methods for detection of water samples positive for coliforms. The number of positive samples detected by each technique is given in the text. Positive samples are defined as at least 1 coliform per 100 ml of the MPN (FT) and MF tests, or a positive reaction in the P-A test.

positive samples than either the MPN (FT) ($p < 0.001$) or the P-A ($p < 0.001$); the P-A detected slightly more positives than the FT ($p = 0.02$).

To obtain a comparison of samples with both low and high coliform densities, the coliform counts for the FT and the MF test were divided into three categories: negative, ≥5, and 1 to 4 (inclusive) per 100 ml. The latter represents an arbitrary figure for identifying systems with low coliform densities. Figure 2 indicates the cross-classified coliform counts for each pair of techniques. These data were analyzed statistically (see below), but one conclusion is obvious: the P-A test was always positive when the MF count was ≥5

A

PA	MPN VALUES 0	1-4	≥5	
−	1160	21	6	1187
+	48	76	172	296
	1208	97	178	1483

B

PA	MF COUNTS 0	1-4	≥5	
−	1167	20	0	1187
+	100	114	82	296
	1267	134	82	1483

C

MPN VALUES	MF COUNTS 0	1-4	≥5	
0	1184	24	0	1208
1-4	53	42	2	97
≥5	30	68	80	178
	1267	134	82	1483

FIG. 2. Cross-classified coliform counts for each pair of microbiological techniques.

TABLE 2. Kappa measures of agreement and their standard errors for pairwise agreement between the three techniques

Comparison	Observed κ (SE)
PA vs MPN (FT) (0 vs ≥1)[a]	0.83 (0.02)
PA vs MF (0 vs ≥1)	0.72 (0.03)
MF vs MPN (FT) (0 vs ≥1)	0.74 (0.02)
MF vs MPN (FT) (overall)[b]	0.59 (0.02)

[a] (0 vs ≥1) indicates negative versus positive.
[b] Classification of samples into negative, 1 to 4 (inclusive), and ≥5 categories.

(Fig. 2B). Furthermore, there were 30 samples where the MPN (FT) value was ≥5, but the MF was negative (Fig. 2C).

The statistical analysis for the data in Fig. 2 is presented as kappa values (see Methods) in Table 2. The agreement between P-A and FT for a simple negative-positive classification (0 versus ≥1) was excellent. Agreement between MF and the other two techniques for the same binary classification was not as strong but still good. Moderate agreement only was found between FT and MF for overall classification of samples into negative, low-coliform (1 to 4), and high-coliform (≥5) categories.

In Fig. 2C the counts below the diagonal are much higher than those above the diagonal: of the 177 total disagreements, 151 (85%) occurred below the diagonal. This indicates that when there was disagreement on any particular sample, coliform counts for FT were usually higher than those for MF. The same pattern of disagreement occurred between the P-A and MF (Fig. 2B): there were 120 disagreements, 100 (83%) of which showed MF negative and P-A positive. It should be noted, however, that agreement between FT and P-A was very strong (Fig. 2A and Table 2). There were fewer (75) overall disagreements; 48 of these (64%) showed P-A positive and FT negative.

The lower sensitivity of the MF test is also apparent from the data in Fig. 1, which compares all three techniques as either positive or negative. There were 63 samples (19% of the positives) for which both the FT and P-A tests together were positive while the MF test was negative. In addition, for 120 samples (36% of the positives) the MF was negative and either the MPN (FT) or the P-A gave positive results. Furthermore, there were only 13 samples for which the MF was positive alone, 7 samples for which the MF and FT alone were positive, and 11 samples where the MF and P-A tests alone were positive.

In summary, both the MPN (FT) and the P-A methods appear to be more sensitive than the MF test. That the FT and P-A strongly agree gives some evidence that the MF could be less sensitive. In addition, the P-A appears to be a more sensitive test than the FT.

A formal analysis of the disagreements between the MF and the P-A and FT techniques is shown in Table 3, using conditional kappa values (see Materials and Methods). When MF ≥ 5, agreement with the FT was excellent, i.e., MPN value of ≥5. When MF ≥ 1, agreement was very good, i.e., P-A positive; MPN value of ≥1. However, when MF = 0, agreement (i.e., P-A negative; MPN = 0) was only moderate. In summary, when the MF technique indicated a positive sample, the other two techniques showed strong agreement; when the MF technique indicated a negative sample, agreement was not as strong. Furthermore, the higher the coliform count for the sample as determined by MF, the stronger the agreement with the FT technique. Very poor agreement was found between FT and MF when MF density was between 1 and 4 (inclusive) (see Fig. 2C and

TABLE 3. Conditional kappa measures of agreement with MF as the standard[a]

MF count	Observed κ (SE)	
	MPN (FT) agreement	PA agreement
0	0.65 (0.03)	0.61 (0.02)
1–4	0.26 (0.02)	N/A[b]
≥1	0.86 (0.03)	0.88 (0.03)
≥5	0.97 (0.04)	N/A

[a] See Materials and Methods for explanation of conditional kappa measures and for the use of MF as the standard for comparison.
[b] N/A, Not applicable.

Table 3): although 110 of 134 (82%) of these samples tested positive by FT, 68 of the 134 (51%) samples had ≥5 coliforms by the FT test. Again, it was seen that the FT consistently read more coliforms for a sample than the MF.

Confirmation efficiency and importance of gas production. A useful screening test should exhibit a high ratio of presumptive positives to confirmed positives. We calculated the percentage of presumptive positive tests that were subsequently confirmed (confirmation efficiency) for each technique (Table 4). The confirmation efficiency of the MF technique was 93%, with 654 of 702 suspected colonies picked from the membrane filters confirmed as coliforms. The confirmation efficiency of the FT technique was 93%, with 1,043 of 1,125 presumptive positive tubes confirmed as coliforms.

The efficiency of the P-A test compared favorably. Of the 316 presumptive positive P-A tests that showed acid (either strong or weak) and gas, 94% were confirmed as coliforms. Two categories of positive P-A tests were noted. The vast majority showed both a strong acid (yellow color) and gas reaction. Of 277 of these tests, only 8 were not confirmed, giving a 97% confirmation efficiency for this category. However, there were 39 borderline tests which had relatively slight color changes (a brownish yellow) with a small amount of gas (4- to 6-mm gas bubble in fermentation tube). Of these 39 cases, 27 were confirmed, giving a 69% confirmation efficiency. In contrast, there were 24 tests in which only acid reactions and no gas production was observed. None of these was confirmed, indicating the critical importance of examining the P-A test for gas production.

A resampling scheme was conducted to assess the validity of our modification of the FT technique. As indicated in Materials and Methods, we omitted the final step of the completed test, in which typical isolated colonies on EMB agar are transferred to LTB. Each of the 15 water systems was resampled an equal number of times. A total of 49 positive samples were obtained for which at least one tube carried through to the entire completed step (5). In this resampling scheme, we subcultured all BGLB tubes positive at the confirmed stage. These samples showed a total of 147 tubes that carried through to the first step of the completed test, where typical colonies were observed on EMB agar. All of these typical colonies were transferred to LTB, the final step of the completed test, and produced gas within 48 h. These results indicate that the observation of typical colonies on EMB agar was a true reflection of the presence of coliforms. Therefore, for the water samples we investigated, our modification of the completed step of the FT procedure was valid.

Organisms isolated from the P-A test. Organisms were isolated and identified from the 37 P-A tests that were positive when the MF and FT tests were negative. The API system for identification and nomenclature was used. Table 5 lists the organisms along with the number of times they were isolated. *Citrobacter freundii* was the most frequently isolated organism, followed by *Enterobacter agglomerans*, and *Serratia plymuthica*. Most (81%) of these 37 presumptive positive P-A tests were noticed on day 2 of incubation, rather than at 24 h. The color of these positives ranged from brown to yellow, and gas was detected in each case. Each of the pure isolates which was designated as a species of the genus *Serratia* was reinoculated into lactose broth to confirm its ability to produce gas from lactose. All these organisms produced gas upon retesting. The identities of the *S. plymuthica* species were confirmed by the Analytab Aerobe Laboratory (Analytab Products, Plainview, N.Y.).

Comparison of the 10-tube FT with the 5-tube FT tests. The FT technique tested in this study used 10 10-ml tubes, as opposed to the FT technique which utilizes only 5 10-ml tubes (1). As a means of comparing the 10-tube with the 5-tube FT, we separately tabulated the number of water samples which would have tested positive if only 5 tubes had been analyzed instead of 10. This comparison was done by separating the 10-tube method into two sets of 5 tubes, consisting of the even- and odd-numbered tubes. Of the 275 water samples positive by FT, both sets of five tubes showed

TABLE 4. Confirmation efficiencies of the P-A, MPN (FT), and MF techniques

Test	Description of test result	No. subjected to confirmation	No. confirmed	% Confirmed
P-A	Strong acid, gas	277	269	97
	Slight acid,[a] gas	39	27	69
	Strong or slight acid, gas[b]	316[b]	296[b]	94
	Strong or slight acid, no gas	24	0	0
MPN (FT)	Tubes with gas	1,125	1,043	93
MF	Metallic-sheened colonies	702	654	93

[a] Slight acid was indicated by a brownish-yellow color.
[b] Sum of the two lines above.

TABLE 5. Organisms isolated from positive P-A tests when the MF and MPN (FT) tests were negative

Isolate[a]	No. of times isolated
Enterobacter agglomerans	6
E. cloacae	3
E. aerogenes	2
Citrobacter freundii	8
Escherichia coli	1
Klebsiella pneumoniae	5
K. ozaenae	1
K. oxytoca	2
Serratia plymuthica	6
S. fonticola	1
S. rubideae	1
S. oderifera	1
Hafnia alvei	1

[a] Identified according to the profile numbers determined by API.

TABLE 6. Positive samples detected by MF, FT, and P-A techniques in each of the 15 water systems

System	No. of positive samples by:		
	MF	FT	P-A
011	18	33	38
021	17	21	22
031	55	57	62
041	0	1	0
051	1	2	2
061	82	100	96
071	6	15	16
081	6	2	5
091	9	12	12
1011	2	1	8
1021	15	24	25
1031	4	2	3
1041	0	4	4
1051	0	0	0
1061	1	1	3

at least one positive tube in 207 (76%) of the samples. On the other hand, there were 68 (24%) samples for which only one set of five tubes showed one or more positive tubes. Thus, assuming an equal distribution of coliform in the tubes, on average 88% of the positives from the 10-tube method would have been detected using only 5 tubes (88% = 76% + 1/2 24%).

Comparison of the three methods in individual water systems. Approximately 100 samples were collected from each system. The results for each individual system are compared in Table 6. Systems 061 and 031 were the most frequently contaminated, and the MF technique compared favorably with the P-A technique in detecting positives in these two systems (Table 6). In contrast, the P-A technique detected many more positives than the MF technique in some of the less frequently contaminated systems. For instance, system 011 was positive 38 times by the P-A but only 18 times by the MF procedure (Table 6).

DISCUSSION

The results indicate that, under the circumstances of our tests, the P-A is much more sensitive than the MF and slightly more sensitive than the FT. There are several factors that may account for this observation.

The MF technique gave negative readings in many samples for which the MPN (FT) and P-A methods were positive. Previous studies have also reported the lower sensitivity of the MF technique in recovering coliforms. Reasons suggested for this failure have included the survival of coliforms on a membrane filter surface compared to survival when in broth (23), the failure to revive injured coliforms or weakened cells (19, 24), or the possibility that the M-endo broth used in the MF test is a selective medium which may be inhibitory to stressed coliforms (3, 4, 24). Inoculation of a sample into an enriched broth-based medium with a prolonged incubation period may enhance the recovery of indicator organisms (3, 24). These organisms may have been injured coliforms that needed more time to grow.

The 10-tube FT technique appears as an optional procedure in the 16th (1985) edition of *Standard Methods for the Examination of Water and Wastewater* (1). This 10-tube technique provides more precise MPN values than the 5-tube FT. With the particular set of drinking water samples that we studied, 12% of the positive samples on average would have been read as coliform negative if the 5-tube FT had been utilized instead of the 10-tube method.

Since this 10-tube FT procedure uses a 100-ml sample of water, it is comparable to the MF and P-A methods, which also use 100-ml samples. The FT was considerably more sensitive in terms of recovery than the MF procedure. It was surprising that the FT was slightly less sensitive than the P-A test, since they are both broth-based methods relying on gas production and are both incubated for up to 48 h. A possible explanation is the slight difference in medium composition between the P-A and FT tests. There is a slightly higher lactose concentration in the P-A (0.75%) than in the FT (0.5%). It has previously been found that isolates anaerogenic in the FT medium (LTB) were aerogenic in a broth containing a slightly higher lactose concentration (12). Members of the genera *Citrobacter*, *Enterobacter*, and *Klebsiella* were most frequently isolated (12). These organisms were also isolated in the present study from the positive P-A tests when the MF and FT were negative. Other possible explanations for the failure of the FT technique to detect coliforms are given in other studies (6, 11, 13, 17, 21).

The P-A test showed a high confirmation efficiency, which is an important characteristic for a screening technique. This high confirmation efficiency was found only in tests that showed both acid and gas production. P-A tests that showed acid but no gas were not confirmed and probably contained lactose-fermenting species other than coliforms (8–10).

Another important characteristic for a useful screening technique is the ease of detection of a positive test. With the P-A test, we found that a large percentage of all positive samples (91%) showed a distinctive yellow color and gas. Clear negatives (77% of all samples) were readily noted by no change in the purple color and no gas. However, there were a small percentage (3% of all samples) which showed only a borderline color change (yellow-brown or brown), but did show some gas production. These were usually true positives, with a 69% confirmation efficiency. However, when no gas was observed, these borderline color changes did not confirm. These findings have important practical implications for the reading of borderline P-A tests in the field. Borderline color changes which showed a small amount of gas represented approximately 10% of our positive P-A tests. Only 69% of these borderlines were confirmed as coliforms. Therefore, it is particularly important to carry all P-A tests with borderline color changes through to the confirmation step before making any decision about the presence of coliforms.

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