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Literature Cover Sheet

IDEXX #: 2B

Title: National Field Evaluation of a Defined Substrate Method for the Simultaneous Enumeration of Total Coliforms and Eschericha coli from Drinking Water: Comparison with the Standard Multiple Tube Fermentation Method

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Date: June 1988
Source: Applied and Environmental Microbiology, Vol 54, No. 6, pages 1595-1601

Topic: Colilert vs. MTF

Highlights:
One of the national collaborative studies that the U.S. EPA approval for Colilert was based on. Split samples from a wide variety of water sources - five utilities representing six U.S. EPA regions- deep and shallow wells, springs, rivers, surface reservoirs -- were tested by multiple tube fermentation and by Colilert used as an MPN format. Heterotrophic suppression of coliform growth was shown in some multiple tube fermentation test tubes, but not with Colilert. This contributed to lower MPN values for multiple tube fermentation in some portions of the study, but statistical analyses showed no significant differences between the two methods.

Bacteria isolated from positive tubes of each method proved to be a bacteria commonly considered part of the total coliform group, and there did not appear to be a significant difference in the distribution of bacterial species for either method. A list of coliform bacteria isolated is given.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1988, p. 1595–1601
0099-2240/88/061595-07$02.00/0

Vol. 54, No. 6

National Field Evaluation of a Defined Substrate Method for the Simultaneous Enumeration of Total Coliforms and *Escherichia coli* from Drinking Water: Comparison with the Standard Multiple Tube Fermentation Method

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Received 21 January 1988/Accepted 18 March 1988

A defined substrate method was developed to simultaneously enumerate total coliforms and *Escherichia coli* from drinking waters without the need for confirmatory or completed tests. It is a new method based on technology that uses a hydrolyzable substrate as a specific indicator-nutrient for the target microbes. No equipment other than a 35°C incubator and long-wavelength (366-nm) light is necessary. To perform the test, one only has to add water to the powdered ingredients in a tube or flask. If total coliforms are present in the water sample, the solution will change from its normal colorless state (no target microbes present) to yellow. The specific presence of *E. coli* will cause the same tube to fluoresce under a longwave (366-nm) UV lamp. The test, called Autoanalysis Colilert (AC), was compared with *Standard Methods for the Examination of Water and Wastewater* 10-tube multiple tube fermentation (MTF) in a national evaluation. Five utilities, representing six U.S. Environmental Protection Agency regions, participated. All water samples came from distribution systems. Split samples from a wide variety of water sources were analyzed for the MPN-versus-MPN comparison. A total of 1,086 tubes were positive by MTF, and 1,279 were positive by AC. There was no statistical difference between MTF and AC. Species identifications from positive tubes confirmed the sensitivity of the AC. A national evaluation of the AC test showed that it: (i) was as sensitive as *Standard Methods* MTF, (ii) specifically enumerated 1 total coliform per 100 ml, in a maximum of 24 h, (iii) simultaneously enumerated 1 *E. coli* per 100 ml in the same analysis, (iv) was not subject to false-positive or false-negative results by heterotrophic bacteria, (v) did not require confirmatory tests, (vi) grew injured coliforms, (vii) was easy to inoculate, and (viii) was very easy to interpret.

There are two standard methods for the enumeration of total coliforms from drinking water. The multiple tube fermentation (MTF) technique provides a most-probable-number (MPN) analysis after growth of total coliforms in liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a CFU/100 ml count (1). Both methods require confirmed and completed tests after the initial observation of a positive primary medium. A complete analysis can require an additional 24 to 72 h for a final result. In neither method is an isolate identified to species or are any members of the fecal coliform group differentiated from the total coliform group (9, 10, 14–16, 21). Both the MTF and MF techniques have been extensively studied and are approved for regulatory monitoring purposes (3, 10, 11, 15, 18, 19, 21, 23).

During a biofilm total coliform regrowth problem in the distribution system in New Haven, Connecticut (8), several inherent properties of the MF and MTF techniques limited the ability of public health officials to make decisions regarding the health risk. Most restricting were the time required to obtain a definitive health-based answer, the inability to differentiate fecal from total coliforms without either the performance of tests specifically for fecal coliform or identification of bacteria, and the subjective nature of interpretation of the analytical methods. In response to these limitations, a new technique was developed, based on technology originally designed to identify microbes by the analysis of their constitutive enzymes. This method uses a hydrolyzable substrate as a defined substrate for only the target microbe(s) one wishes to enumerate (S. C. Edberg and M. K. Edberg, Yale J. Biol. Med., in press). The technology is designated autoanalysis, because a color change is produced by the target microbe(s), with no need for confirmatory tests or technologist labor. To perform the test, one need only add water to the powdered formula in a tube or flask and incubate. No equipment other than an incubator and 366-nm lamp is necessary. Specific color changes denote the presence of the target microbe(s). Only the target microbes, total coliforms (yellow) and *Escherichia coli* (fluorescence), produce color changes during the test period.

In applying the autoanalysis technology to microbial water analysis, we hoped to achieve the following goals: (i) to specifically enumerate 1 total coliform per 100 ml in a

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TABLE 1. Characteristics of participating water utilities

Geographical area	Water source	Type of utility	Population served
California, New Mexico, Arizona	Well, ground, surface	Private	>250,000
Southwestern Pennsylvania	Surface, river	Private	>530,000
Connecticut	Well, ground, surface, mixed	Municipal	400,000
Northern New Jersey	Well, ground, surface, river	Private	250,000
Northern Georgia	Surface	Municipal	250,000

maximum of 24 h, (ii) to simultaneously, specifically enumerate 1 *E. coli* per 100 ml in the same test, (iii) to not be affected by heterotrophic plate count (HPC) organisms found in drinking water, (iv) to not require confirmatory tests, (v) to grow injured coliforms, (vi) to be easy to inoculate, and (vii) to be very easy to interpret.

The autoanalysis method was compared in a national evaluation with the 10-tube MTF test described in *Standard Methods for the Examination of Water and Wastewater* (1). The protocol explicitly followed was that of the U.S. Environmental Protection Agency (E.P.A.) Environmental Monitoring Support Laboratory for certification of an alternative method (6). Collaborative water utilities were chosen to reflect all sources of drinking water and to represent a variety of geographical locations, treatment techniques, and water quality conditions.

MATERIALS AND METHODS

Participants and samples. Five utilities, representing six U.S. E.P.A. regions, participated in the national evaluation (Table 1). The utilities ranged from those serving a single geographical area to those serving large numbers of small community water systems over three states. Water sources included deep and shallow wells, springs, rivers, and surface reservoirs. All water samples were obtained from distribution systems by the participating utilities; however, an effort was made to obtain water from locations most likely to yield positive samples, such as dead ends, storage reservoirs, and known problem sites. In accordance with U.S. E.P.A. Environmental Monitoring Support Laboratory guidelines, sites were permitted to mix small amounts of treatment effluent or raw water with a large volume of distribution water to achieve positive samples with natural microbial populations (6). Samples were also collected during periods of flushing. These water samples were not necessarily those used for routine monitoring for regulatory purposes. Sections of two of the utilities had been experiencing total coliform regrowth problems.

Water samples were collected, transported, and stored in strict accordance with the guidelines described by *Standard Methods* and the U.S. E.P.A. (6, 13). Either sterile polymethylpentene or glass flasks containing sodium thiosulfate were used to collect the samples.

AC test. The defined substrate method, called Autoanalysis Colilert (AC), was prepared for us as 10-tube MPN tests (Access Medical Systems, Branford, Conn.). The powder formula contained, per liter, the following: $(NH_2)_4SO_4$, 5 g; $Mn(SO_4)_2$, 0.5 μg; $ZnSO_4$, 0.5 μg; $MgSO_4$, 100 mg; NaCl, 10 g; $CaCl_2$, 50 mg; KH_2PO_4, 900 mg; Na_2HPO_4, 6.2 g; Na_2SO_3, 40 mg; amphotericin B, 1 mg; *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG), 500 mg; 4-methylumbelliferyl-β-D-glucuronide (MUG), 75 mg; and Solanium, 50 mg. Solanium is a plant extract mixture that acts as an emulsifier.

All ingredients were obtained from Sigma Chemical Co. (St. Louis, Mo.), with the exception of Solanium (Access Medical Systems). Each test tube (13 by 100 mm) was aseptically filled and heat disinfected at 70°C for 0.5 h. The quality control parameters were based on the following criteria: the test must detect 1 CFU/100 ml in 24 h in environmental isolates of *E. coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Citrobacter freundii* and not be subject to interference by at least 10,000 CFU of *Pseudomonas*, *Flavobacterium*, and *Aeromonas* spp. per ml. A high proportion (1.5%) of tubes was chosen for quality control. Each of the test species was diluted to 1 CFU/100 ml, and an MPN analysis was performed. In addition, species of heterotrophs at concentrations of 10,000/ml were mixed with these bacterial concentrations in separate test tubes. Positive (yellow or yellow and fluorescent) had to be observed in 24 h.

The AC method was performed as follows: 10 ml of water was added to each tube, dissolving the powder after agitation and producing a colorless solution. The test tubes were incubated at 35°C for 24 h. Development of a yellow color after incubation indicated the presence of total coliforms in the test tube. Each positive total coliform test tube was exposed to a hand-held fluorescent (366-nm) light (Edmund Scientific Co., Barrington, N.J.) Fluorescence in the test specifically denoted the presence of *E. coli*. Therefore, a separate MPN analysis was obtained for total coliforms and *E. coli* by the 10-tube AC method. It was designed so that no confirmatory or completed tests need be performed with the AC test.

MTF test. The 10-tube MTF test was performed with each tube containing 10 ml of double-strength lauryl tryptose broth (LTB; Difco Laboratories, Detroit, Mich.). Positive tubes were confirmed in brilliant green lactose bile broth (BGLB broth; Difco) (4). The number of coliforms per 100 ml was estimated from a 10-tube MPN table (1). Tests that confirmed initial positive results were included in the data base.

HPC. An HPC was determined for each water sample according to *Standard Methods* using R2A agar incubated at 35°C for 48 h (1).

Evaluation protocol. Sufficient water was collected from each location to perform a simultaneous split sample analysis by the AC test and the MTF technique. For each water sample, the following microbiological analyses were performed: HPC, a 10-tube AC MPN analysis, and an MTF test (Fig. 1). Within the guidelines of the *Handbook for Evaluating Water Bacteriological Laboratories* (13), samples were prescreened for the presence of coliforms before being analyzed. The U.S. E.P.A. equivalency protocol called for four replicate analyses of each method from each sample. Accordingly, four rows of 10 LTB tubes and four rows containing 10 AC tubes were inoculated from each 1-liter water sample. LTB tubes were examined for positivity according to *Standard Methods* (1). Confirmation tests were also performed according to *Standard Methods* (1). The AC tube was examined at 24 h. Any yellow color was designated

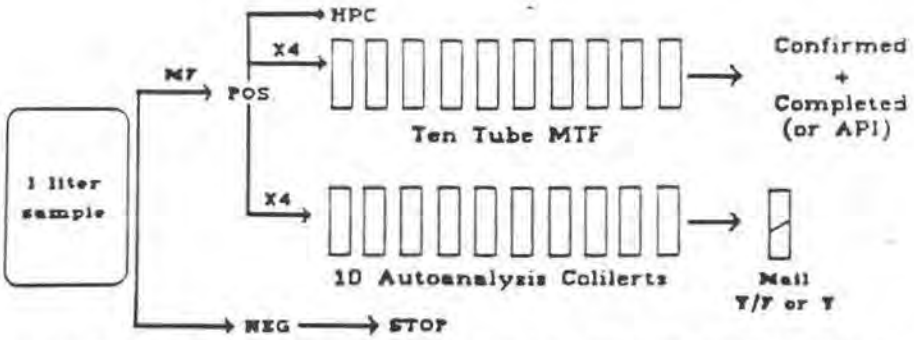

FIG. 1. National evaluation comparing the defined substrate method (AC) MPN and *Standard Methods* MTF. MF, Screening membrane filter; ×4, four repeats from the same sample; Y/F, yellow/fluorescent AC test; Y, yellow only AC test; Mail, subculture sent to the reference laboratory for identification as to species; POS., positive; NEG, negative.

as positive for total coliforms; fluorescence denoted *E. coli*. From at least one positive MTF or AC tube per row, a subculture was made to ensure the presence of a total coliform by identifying the bacterial isolate(s) as to species. All bacterial identifications were performed by the API 20E system (Analytab Products, Plainview, N.Y.) (7).

Statistical analysis. The data were analyzed by using several statistical tests. The Statistics Section of the Department of Epidemiology of Yale University analyzed much of the data. First, by using the criteria of the U.S. E.P.A., *t* tests were performed. The *t* tests were calculated for each participating utility and for the utilities as an aggregate. The *t* tests were performed in two parts: first, by considering that the samples consisted of four replicates of 10 tubes for each method and second, by considering each analysis to be a simple 40-tube MPN analysis (2, 12).

Second, since the *t* test is more appropriate to chemical than bacterial analysis, in which values are continuous rather than incremental, the data were also examined by chi-square analysis. Two chi-square tests were calculated: the Mantel-Haenzel and Pearson (2, 12). The Mantel-Haenzel test generates a separate 2 × 2 table from each replicate, with all statistics from all tables combined to produce a single test statistic. The Pearson test combines all samples into one 2 × 2 table (2, 12).

Third, the two methods were analyzed by regression analysis (2, 12). All statistics were generated to include and exclude all tubes, positive and negative.

RESULTS

Comparison of *Standard Method* MTF and AC test. (i) Number of tubes positive. A total of 46 distribution water samples were analyzed from the five sites. A total of 1,086 tubes were positive by MTF, and 1,279 were positive by AC. For the MTF analysis, the median number of tubes positive per 40 inoculated from a sample was 24, the mean was 23.6, and the standard error was 2.0. For the AC test, the median was 34.5, the mean was 24.8, and the standard error was 2.1.

The number of positive tubes for each of the four MPN rows for each utility is presented in Table 2. This measure allows an overall comparison between the two methods, and it permits this correspondence, regardless of the variation in an individual analysis.

(ii) Effect of HPC on total coliform recovery. Total HPCs ranged from 3 bacteria per ml to greater than 4,000/ml. Heterotrophic interference occurred in four fermentation test analyses. MTF tubes did not yield a coliform on subculture from these positive tubes. In each of these cases, the HPC was greater than 1,500/ml. HPC suppression of coliform growth resulted in a loss of between 17 and 24 tubes out of a possible 40 per sample (Table 3). Positive autoanalysis tubes did not demonstrate the growth of heterotrophs when samples were taken for subculture but did yield a coliform.

Statistical analyses. (i) *t* tests. The *t* test analysis is used by the U.S. E.P.A. to evaluate a proposed method for certification as an alternative testing procedure. *t* statistics were calculated for all sites as an aggregate and for each site individually, first excluding samples which yielded either all positive tubes or all negative tubes. There was no statistical difference in utilizing this measure between MTF and the AC, with an overall P value of <0.001. The *t* test was also calculated including samples that yielded either all positive tubes or all negative tubes. By paired *t* tests, which included samples yielding all positive or negative tubes, *Standard*

TABLE 2. Comparison of *Standard Methods* and AC MPN results from split transmission water samples[a]

Row	No.[b] of positive tubes from:									
	Site A (11)[c]		Site B (11)		Site C (8)		Site D (8)		Site E (8)	
	Standard Methods	AC	*Standard Methods*	AC	*Standard Methods*	AC	*Standard Methods*	AC	*Standard Methods*	AC
1	74	72	52	72	66	67	34	46	47	61
2	75	73	44	78	64	71	37	39	52	59
3	74	68	49	78	69	72	31	43	50	57
4	63	73	56	77	64	71	33	43	52	54

[a] MPN tests were inoculated from split samples to 4 rows containing 10 tubes each (see Materials and Methods).
[b] The number of positive tubes from the 10 tubes inoculated in that row, multiplied by the number of rows inoculated.
[c] Number of distribution water samples tested at the site. Site C includes two geographically close utilities in the same U.S. E.P.A. region.

TABLE 3. Recovery of HPC-suppressed *Standard Methods* tube by AC

Sample no	*Standard Methods*		No. positive by AC	HPC (CFU/ml)
	No. positive	No. suppressed		
16	11	17	28	>3,000
22	17	22	39	>4,500
30	22	18	40	>1,000
42	2	24	26	>2,500

Methods MPN gave a mean and standard deviation of 23.609 ± 13.881 (compared with 27.804 ± 14.120 by AC), with 46 paired observations; the t statistic was −4.196, with 45 degrees of freedom and 0.000 significance. Although the AC method appeared to yield somewhat higher MPN values (Table 2; also see Fig. 2), there was no difference between the two methods by this anaylsis, with a P value of <0.001.

(ii) Chi-square analyses. The two methods were also compared by two chi-square statistics. First, the Pearson chi-square test for the overall proportion of positive tubes was determined. By this analysis, three of the utilities demonstrated that the AC was more sensitive than the *Standard Methods* MTF ($P > 0.05$); there was a positive bias, with slightly greater AC sensitivity overall. The Mantel-Haenzel test compared the two methods by determining the difference between the positive tubes for each method and subtracting from it the expected number of positive tubes and analyzing the differences. This statistic yielded the same results as the Pearson test (Table 4).

(iii) Regression analysis. Data were analyzed in simple and multiple regression formats with the AC MPN being the x variable and *Standard Methods* MTF being the y variable. The two methods agreed with each other to yield an r value of 0.883 and an r^2 value of 0.779 (Fig. 2). The adjusted r^2 value was 0.774, and the standard error was 6.59 (Fig. 2). Analysis of variance demonstrated no difference between the two methods, with the F test yielding a P value of 0.001. The beta coefficient table for the simple regression yielded a slope of 0.868, a standard error of 0.07, a standard value of 0.883, a t value of 12.47, and a P value of 0.0001. Therefore, there was no difference by regression analysis between the *Standard Methods* MTF and AC MPN, although some bias in favor of the AC test was evident.

(iv) Precision. For each of the two methods, assumptions of normality and homogeneity of sample variance were performed. The response analyzed was the proportion of positive samples under an arcsin transformation. For the locations meeting these requirements, an F test was used to analyze method precision. If not, Scheffé test was employed. Precision analysis revealed that the AC test was significantly more precise than MTF in two of the five sites (Table 5 courtesy of R. Freyberg and T. Grady, U.S. E.P.A. Environmental Monitoring and Support Laboratory).

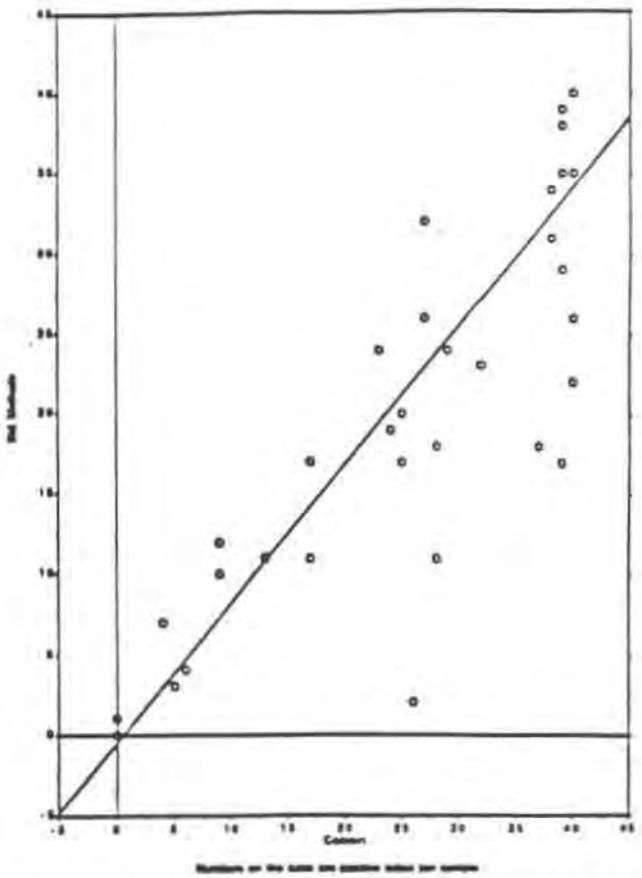

FIG. 2. Comparison of the AC and *Standard Methods* MPN by regression analysis. Shown is the simple regression analysis ($y = 0.868x - 0.524$, with $r^2 = 0.779$). There was no difference when the comparison was made by multiple regression analysis.

(v) Likelihood ratio test. The likelihood ratio test compares the estimates of mean bacterial density obtained from different sets of data (20). If one assumes that the distribution of the number of positive responses at a given dilutional level for the MPN method is binomial, then the likelihood function may be calculated taking into account the volume of diluent, factors, etc. Calculation of the likelihood ratio (Λ) from the individual likelihood function $L(\lambda)$ by the formula $\Lambda = L(\lambda)/L(\lambda)\ldots L_x(\lambda_x)$ showed a slight bias in favor of the AC method, $-2 \ln\Lambda$ significant at 0.5%.

TABLE 4. MPN method comparisons by paired t tests excluding samples yielding all positive or negative tubes

Site	N[a]	Mean difference		SE		T		Probability of |t|	
		Group 1[b]	Group 2[b]	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
All	35	7.68	3.88	1.59965	0.90587	4.80	4.28	0.0001	0.0001
1	6	12.77	4.81	4.48141	2.26522	2.85	2.12	0.0159	0.0272
2	6	5.95	2.93	2.59920	1.60107	2.29	1.83	0.0708	0.1268
3	5	8.31	2.99	5.41892	2.28604	1.53	1.31	0.2228	0.2824
4	10	11.74	7.95	3.35564	1.81269	3.50	4.38	0.0067	0.0018
5	8	0.63	−0.18	1.25953	1.00181	0.50	−0.18	0.6326	0.8616

[a] Number of tests, excluding all positive or negative tubes.
[b] Group 1 considered the samples as one replicate of 40 tubes, whereas group 2 considered the samples as four replicates of 10 tubes.

TABLE 5. Precision of the two methods by source

Location	P value	
	F test	Scheffe test
Cobb County, Ga.		0.2266
Monterey, Calif.	0.0131*	
Tinton Falls, N.J.		0.1534
New Haven, Conn.	0.0006*	
Pittsburg, Pa.		0.5725

* *Standard Methods* MTF was significantly less precise than the AC method

Performance characteristics. The original performance goals for which the AC was designed were met, and additional benefits became apparent. The collaborators felt that the yellow color produced by the total coliforms and the fluorescence generated by *E. coli* were distinct, easy to read, and apparent within the time framework of the test (24 h). They also noted that total coliform counts greater than 25/100 ml yielded results in 16 to 18 h. Separate MPNs for total coliforms and *E. coli* were produced simultaneously. All participants affirmed the ease of one-step inoculation and clarity of interpretation of positive test results. They substantiated the autoanalytical nature of the test by verifying the goal that confirmatory tests need not be performed because of the specificity of the ingredients.

Bacteria isolated. Bacteria isolated from each split sample were identified as to species (Table 6). Bacteria commonly considered part of the total coliform group were found from both *Standard Methods* MTF and AC tubes. *K. pneumoniae* was the most common species isolated, followed by *E. cloacae* and *C. freundii*. There were mixed cultures of total coliforms present in both MTF and AC tubes. The higher the bacterial count in the water sample, the greater the number of species found. There did not appear to be a significant difference in the distribution of bacterial species in either method.

DISCUSSION

The AC test is based on a new technological principle in which an indicator-nutrient is an essential food source for the target microbe (Edberg and Edberg, in press). Because a chromophore is released each time an indicator-nutrient molecule is hydrolyzed, color production is directly related to the growth of the target bacteria. In the application of this technology to drinking water analysis, the indicator-nutrients are ONPG (for total coliforms) and MUG (for *E. coli*). Unlike classical enzyme assays, which use ONPG or MUG only as substrates for constitutive enzyme tests, the autoanalysis technology uses these compounds both as an essential growth substance and as an indicator system (color formation and fluorescence). Therefore, there is not a direct correspondence between tests in which ONPG and MUG are used as substrates in constitutive enzyme tests (e.g., Colifern; Millipore Corp., Waltham, Mass.) and the growth-dependent AC test. For example, species in the genera *Aeromonas*, *Pseudomonas*, or *Flavobacterium* that may be positive in a constitutive ONPG or MUG enzyme assay will not be positive in the 24-hour growth-based AC test, unless a high density (≥20,000 bacteria per ml) is present (Edberg and Edberg, in press).

In conventional medium, several secondary reactions must occur before a change in the indicator is visible. The target microbe must transport the substrate (e.g., lactose) through the cell membrane, transform the substrate to glucose, metabolize glucose through the glycolytic cycle to pyruvate, and then convert pyruvate to the desired end product, either acid or gas. Because conventional testing requires the microbe to go through many steps to yield a positive visible endpoint, a number of anomalous results may occur, such as false-negative gas producers (i.e., anaerogenic *E. coli*) or false-positive tests (i.e., acid from lactose by some clones of *Aeromonas hydrophilia*). ONPG-positive noncoliforms do not yield a positive AC test, because the formula does not support their metabolism. Therefore, the detection of the β-galactosidase system of heterotrophs will occur only at extremely high microbial concentrations (>20 to 100,000/ml). This phenomenon will be seen with approximately 10% of members of the genus *Aeromonas*.

The specificity of the defined substrate autoanalysis technology eliminates the need to perform confirmatory and completed tests. In conventional methodology, transfers from the original positive tube or plate must be made into BGLB broth for confirmatory testing and a second transfer must be made to solid or liquid medium for a completed test. As substantiated in this national evaluation (Table 6), the 24-h AC test is as specific as the multiple day confirmed and completed *Standard Methods* MTF analysis.

Species identification from positive tubes confirmed the specificity of the AC test. From positive AC tubes, members of the total coliform group were recovered. Therefore, yellow test tubes were only the result of the growth of the target microbe(s). Likewise, *E. coli* was routinely recovered from tubes that fluoresced.

In addition to specificity, the autoanalysis technology was shown to have equal sensitivity with *Standard Methods* MTF. *Standard Methods* MTF testing will enumerate one, (≤1.1 for a 10-tube test) total coliform per 100 ml (1). The AC test also demonstrated this level of sensitivity, even in the presence of high concentrations of heterotrophic bacteria. It has been noted that current methods are subject to heterotroph interference above 500 to 1,000 HPC per ml (4). Because the AC test does not support the growth of heterotrophs in the densities found in water, these bacteria did not interfere.

There did not appear to be a loss in sensitivity due to the inability to enumerate injured coliforms. Previous laboratory

TABLE 6. Species of total coliforms isolated*

Species	% of all isolates belonging to species by method:	
	Standard Methods	AC
Klebsiella pneumoniae	31	28
K. oxytoca	3	6
Enterobacter agglomerans	19	16
Enterobacter species	3	7
E. cloacae	10	11
E. aerogenes	1	1
Citrobacter freundii	16	12
Serratia plymuthica	3	4
S. fonticola	1	1
S. rubidaea	3	4
S. odorifera	2	3
Hafnia alvei	1	1
Escherichia coli	1	1
CDC groups	3	4
Unidentified *Enterobacteriaceae*	3	3

* All isolates confirmed in BGLB.

analysis demonstrated that the AC test can enumerate injured coliforms within 24 h, although the intensity of color production is less, compared with that of noninjured coliforms (Edberg and Edberg, in press).

Several benefits other than those described above were noted by participating water utilities during the national evaluation. The autoanalytical nature of the method made it much more practical and efficient to perform than current coliform methods. The participants felt that interpretation of the AC test was less subjective than that of the *Standard Methods* procedure. It was much easier to see whether a yellow color or fluorescence was produced than to decide whether LTB tubes were positive. The determination of gas was often subjective and not enhanced by an additional 2 to 4 h of incubation. Because the colors were distinct, it was possible that a moderately trained individual could interpret the AC test. Furthermore, since there were no additional tests needed, an analysis did not extend through weekends, which could result in a delay of 2 to 5 days in obtaining definitive results. Also, in contrast to other methods in which weekends added 2 or more days to the analysis, several participants said that they would be willing to test water on Friday and have someone briefly stop in on Saturday to read the results, thus adding only 1 day to the testing regimen. Virtually any water utility employee could accurately read and record the AC test results.

Because the ingredients are in powder form and have an unrefrigerated shelf life of at least 1 year, the participants felt that they could stock a large number of tubes. This would enable the utilities to respond quickly to either changes in the work flow or emergencies.

A strength of the AC test is its applicability to use by the small utility. First, the AC test can be inoculated in the field. Once the formula is hydrated, the bacteria begin to grow, although more slowly at ambient than incubator temperature. Moreover, one avoids any change in the bacterial composition of the water sample during storage and transport. After incubation, the characteristic nature of the colors makes it possible for the small-utility operator to determine the presence of both total coliforms and *E. coli*. This immediate reading of results could eliminate, with state approval, the inherent delay in sending samples to commercial laboratories. Prolonging sample transit times results in questionable or unacceptable results (6). In addition, significant monetary savings can be realized by the small utility, because their only cost for bacteriological water monitoring is the cost of the test itself. If state certifying agencies require independent verification of the test, the small utility can forward the AC tube to a reference laboratory. The target microbes remain viable in the test for at least 7 days and can be analyzed by another laboratory, if required. The double-strength lactose broth (DSLB) can also be inoculated in the field. However, the determination of a positive result from DSLB is much more subjective than the reading of an AC tube. Furthermore, once color forms in the AC tube it is permanent; extended incubation of the DSLB can result in changes in the medium which make it difficult to interpret.

The cost of the AC test for a single water sample is between 20 and 100% less than *Standard Methods* MTF testing, depending upon whether the utility performs the analysis itself or sends it to an outside laboratory (commercial or regulatory). The participants felt that the major cost saving associated with the test lies in decreased labor and better quality control.

The AC test provides the simultaneous detection of the major fecal coliform, *E. coli*, for no additional cost or effort. Current Safe Water Drinking Act Regulations do not currently require the routine analysis for this species. However, the new regulations may mandate it (11). It is generally thought that *E. coli* is a much more specific indicator of the fecal pollution of freshwater than are total coliforms. The total coliform group is used now, because it has been technically difficult to analyze water directly for this species (5). Public health authorities generally feel that the absence of *E. coli*, even when distribution water has total coliforms in a biofilm regrowth situation, indicates that the finished water is acceptable for consumption (17). Therefore, the ability to simultaneously assay for both total coliforms and *E. coli* is an added dimension to water analysis not previously available. It will provide utilities an immediate measure of whether a sample has been subject to fecal contamination. The utility would not have to perform fecal coliform analyses or bacterial identifications on colonies after subculture of the primary test, as they do now. This additional testing could delay a definitive answer for several days, an undesirable situation engendered by the current available technology. In many cases, additional water samples have to be tested if one wished to pursue a possible pollution event further to determine whether *E. coli* was present in the positive sample; with the AC method, this would not be necessary. The analyst would know whether total coliforms, for regulatory purposes, and *E. coli*, for public health purposes, were present in the same sample.

It should be noted that the AC test has specific limitations. First, while it has been found to be refractory to the density of heterotrophs encountered in the national evaluation, in the laboratory one species of bacterium, *A. hydrophilia*, was found to yield a positive endpoint (yellow color) after 32 h of incubation at very high densities. A total of 20 *Aeromonas* isolates were tested, and a wide range of activity was observed; however, 2 isolates showed positive total coliform reactions at inoculation densities of 20,000/ml at 32- and 36-h incubation, respectively (Edberg and Edberg, in press). While these *Aeromonas* concentrations are unlikely to be encountered in drinking water, and the test should not extend beyond a 24- to 28-h incubation period, it is recommended that any AC test incubated for more than 30 h and then noted as positive should be confirmed by a BGLB broth, other *Standard Methods* confirmation, or species identification.

Isolates of *Pseudomonas* and *Flavobacterium* species did not demonstrate positive total coliform or *E. coli* tests at concentrations up to 50,000 bacteria per ml. Petzel and Hartman (22) did report *Flavobacterium* sp. isolates that yielded a positive constitutive enzyme MUG test from bacterial colonies. These were tested (courtesy of Eugene Rice, U.S. E.P.A.) and did not yield a positive result until densities unlikely to be found in drinking water (≥100,000 bacteria per ml) were reached (unpublished results). It appears that at very high bacterial densities, the AC test may act as an enzyme assay and become positive, not through a growth mechanism, but by direct enzyme measurement. Therefore, the AC test should not be used as a confirmatory test from either MTF broths or colonies from plates.

Second, laboratory testing has shown that injured coliforms exhibit a longer lag phase than normal coliforms in the AC test (Edberg and Edberg, in press). It was found that once the injured coliforms repaired themselves and entered log phase they grew as rapidly as normal coliforms. Practically, this meant that the intensity of yellow color produced at 24 h by injured coliforms may be less than that produced by normal coliforms. If kept between 2 and 4 h longer than

the 24-h incubation period, the injured coliforms achieved the same color level as normal total coliforms. Therefore, it is recommended that if no color is noted at 24 h, the result should be considered negative; if any yellow color is noted, the result should be considered positive. If the analyst is unsure of color formation, he or she should reincubate the test for an additional 2 to 4 h and examine it for an increase in yellow color. This additional incubation period will also account for the slightly yellow color that some waters may have due to humic material, because these tubes will not increase their color intensity and can be classified negative.

Third, although the AC determines the presence of *E. coli* simultaneously with total coliforms, this should not be considered the same as a fecal coliform test. *E. coli* makes up approximately 90% or more of fecal coliforms, but other bacteria, notably *K. pneumoniae*, may also fall into this category if it grows at 44.5°C in E. C. broth with the production of gas (1). Therefore, if *E. coli* is found by the AC test, one may consider the sample to be fecal coliform positive; however, if *E. coli* is not detected, there may still be fecal coliforms in the sample.

Lastly, the national evaluation of the AC test was limited by design to drinking water distribution samples. If the test is to be applied to a different water source, such as storm runoff, marine waters, waste effluents, etc., the user should first establish the efficacy of the test in the environment.

In summary, the AC test can simultaneously detect total coliforms and *E. coli* from a water sample within 24 h. No confirmatory or completed tests need be performed. HPC bacteria do not interfere in densities likely to be encountered. All one does is add water to the tubes and incubate them for a maximum of 24 h. Interpretation of the endpoints, yellow for total coliforms and fluorescence for *E. coli*, is distinct. A national evaluation conducted according to U.S. E.P.A. protocol demonstrated that this test was equivalent with *Standard Methods* MTF. The AC is less costly than *Standard Methods* MTF and requires considerably less labor. It allows small utilities to test as accurately as large ones, further ensuring safe water. The method is currently undergoing national evaluation as a presence-absence test, since this format may be mandated for regulatory purposes in the future (11).

ACKNOWLEDGMENTS

We thank Ramon Lee for his helpful discussions during the course of this project. Edwin Geldreich, U.S. E.P.A., is thanked for his review of the manuscript. This study was reviewed by a project advisory committee of Joyce Kippin, Ipswich, Mass., Richard Tobin, Health and Public Welfare, Canada, and Donald J. Reasoner, U.S. E.P.A.

This work was supported, in part, by the U.S. E.P.A. through a cooperative agreement (CR811335) with the American Water Works Association Research Foundation.

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ERRATUM

National Field Evaluation of a Defined Substrate Method for the Simultaneous Enumeration of Total Coliforms and *Escherichia coli* from Drinking Water: Comparison with the Standard Multiple Tube Fermentation Method

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Volume 54, no. 6, p. 1596, column 1, paragraph 4: "$Mn(SO_4)_2$, 0.5 μg" should read "$Mn(SO_4)_2$, 50 mg"; "$ZnSO_4$, 0.5 μg" should read "$ZnSO_4$, 50 mg"; "Solanium, 50 mg" should read "Solanium, 500 mg."