IDEXX
Literature Cover Sheet

IDEXX #: 2A

Title: National Field Evaluation of a Defined Substrate Method for the Simultaneous Enumeration of Total Coliforms and Eschericha coli from Drinking Water: Comparison with Presence - Absence Techniques

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Topic: Colilert vs. Presence Absence Test

Highlights:
One of the national collaborative studies that U.S. EPA approval for Colilert was based on. Seven water utilities representing a wide geographical and hydrological spectrum participated in a study of Presence - Absence methods including the Standard Methods (membrane filtration, multiple fermentation , P-A Broth) and Colilert.

702 split samples were tested; 322 were positive by Standard Methods and 324 were positive by Colilert. 380 were negative by Standard Methods and 378 were negative by Colilert. The overall agreement was 94%.

Of the disagreements between the two methods (20 samples were positive by Standard Methods but negative by Colilert; 22 samples the reverse) 90% occurred when the total coliform count was less than 10/100 ml. The majority of these disagreements probably represented the uneven distribution of bacteria within the split sample, resulting in sampling error. When comparing methods on split samples in which the coliform level is very low, it is common to get positives with one method and not the other due to the uneven microbial distribution.

By subculture and identification, each time a Colilert test was yellow, a total coliform was present; when a test fluoresced, E. *coli.* was isolated. A list of coliforms isolated during the study was presented.

* Refer to page 1004 paragraph 3 Table 1, page 1005 paragraph 2, page 1006 paragraph 3, page 1007 paragraph 3 Table 5.

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National Field Evaluation of a Defined Substrate Method for the Simultaneous Detection of Total Coliforms and *Escherichia coli* from Drinking Water: Comparison with Presence-Absence Techniques

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A defined substrate method was applied to drinking water to simultaneously enumerate total coliforms and total *Escherichia coli* directly from samples. After incubation at 35°C for 24 h, the development of yellow in an initially colorless solution was specific for total coliforms; fluorescence at 366 nm in the same tube(s) or vessel demonstrated the presence of *E. coli*. No confirmatory or completed steps were necessary. Known as autoanalysis colilert (AC), this method was constituted as a presence-absence test and compared with the methods described in *Standard Methods* (SM) in the P-A format. Seven water utilities representing a wide geological and hydrological spectrum participated in the evaluation. A total of 702 split drinking water samples were analyzed. Of these, 358 were negative in both tests (SM− and AC−); 302 were positive (SM+ and AC+); and 42 were mixed (SM+ and AC−, 20; AC+ and SM−, 22). The overall agreement rate was 94%. Comparison of the SM and AC results by nonparametric statistics demonstrated no differences. Heterotrophic plate count bacteria exerted no discernible effect on the AC test. By subculture, each time the AC test was yellow, a total coliform was present; when the test was fluorescent, *E. coli* was isolated.

Current Safe Drinking Water Act regulations require the analysis of potable water for total coliforms (15), a group of closely related bacteria in the family *Enterobacteriaceae*. Two quantitative methods are presently certified for this analysis, the multiple-tube fermentation (MTF) and the membrane filter (MF) techniques (1, 15). Both of these procedures need verification of first-step presumptive positives by multistep and confirmed tests. Therefore, a water analysis may require from 2 to 4 days (1, 3).

Present enumeration techniques suffer from several inherent limitations. First, estimates of coliform density from a single sample may show variability (14, 20). Second, coliform densities may significantly change from the time the sample is collected until it is processed (20). Third, the MTF method uses a 50-ml sample and is not sensitive enough to enumerate 1 total coliform per 100 ml (16). To address these shortcomings, the Environmental Protection Agency (EPA) proposed a frequency-of-occurrence monitoring approach. Also known as the presence-absence (P-A) concept, this approach determines whether total coliforms are present or absent in a given sample but does not estimate their densities. If an adequate number of samples is examined, the percentage that contains total coliforms provides an estimate of the frequency of occurrence of these indicator bacteria in the distribution system (5, 23–25). The proposed EPA regulations for coliforms in drinking water would replace enumeration with P-A analyses and allow up to 5% positive samples per month (17). The EPA proposed use of the following analytical techniques for determining the presence of coliforms: P-A, MTF, or MF and a 100-ml Colilert test (16, 17). The proposed regulations also require testing a positive total coliform culture for the presence of fecal coliforms (1) or *Escherichia coli* (16, 17).

Clark, in Ontario, Canada, has been using a single-bottle P-A broth for many years to monitor distribution water for total coliforms (6, 7). His method uses a lactose-enriched MTF broth with a pH indicator (8). During the decades it has been used in Ontario, Canada, it has performed well (9). Jacobs et al., comparing the MF, 10-tube MTF, and single-100-ml MTF bottle P-A techniques in small community water systems in Vermont and New Hampshire, showed that the MTF detected 82%, the 100-ml P-A bottle detected 88%, and the MF detected 64% of all total-coliform positives from split samples. They found that the P-A test was able to detect the common species of total coliforms, including *Escherichia*, *Klebsiella*, *Enterobacter*, *Serratia*, and *Hafnia* species (21). A recent project sponsored by the EPA compared the single-vessel P-A test with the MF and 10-tube MTF methods in 10 small water systems in western Oregon. This study also found that the P-A test detected more total-coliform-positive samples than either MF or MTF (4). Those investigators concluded that the current 100-ml MF and 5-tube MTF were not adequate to detect either the incidence or density of total coliforms in potable water (4). In England,

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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
New England	Surface	Municipal	22,000
New York City	Surface	Municipal	8,000,000
Southern Ohio	River	Municipal	750,000
Washington State	Well, ground	State	>1,000,000

a 100-ml P-A test consisting of a 300-ml glass bottle with 100 ml of glutamate broth has been used for several years for the decentralized testing of distribution water (19). All available methods test only for the single component, total coliforms.

The autoanalysis Colilert (AC) method is an application of a defined substrate technology originally developed to elucidate specific species and groups of the family *Enterobacteriaceae* in urine samples (13). It can detect and enumerate total coliforms and *E. coli* simultaneously, directly from a drinking water sample. The AC method was evaluated as an enumeration most-probable-number test according to the Environmental Support and Monitoring Laboratory protocol of the EPA and found to be equivalent to currently approved methods of the EPA (11). Levels of heterotrophic bacteria as high as 7×10^5/ml encountered during the study did not show interference (11).

To determine the sensitivity and specificity of the AC test in the frequency-of-occurrence format, it was constituted as a P-A test and compared with the quantitative methods described in *Standard Methods* (SM; 1) for the MTF, MF, and P-A tests used as P-A tests. A wide variety of geologically diverse surface and groundwater samples were tested. The P-A comparison of AC versus SM followed the guidelines of the Environmental Support and Monitoring Laboratory protocol for certification of a proposed method as an acceptable alternative (10).

MATERIALS AND METHODS

Participants and samples. Eight utilities representing seven EPA regions participated in the national evaluation (Table 1). The utilities ranged from those serving a single metropolitan area to those serving large numbers of small community water systems in three states. Water sources included deep and shallow wells, springs, rivers, and surface reservoirs. All water samples were obtained from potable 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 some cases a small amount of chemically and biologically defined source water was added to a large sample of distribution water to ensure that positives were obtained. Samples were also collected during periods of distribution system flushing. These water samples were not necessarily those used for routine monitoring for regulatory purposes. Two of the participating utilities had been experiencing biofilm regrowth in their distribution systems.

Water samples were collected, transported, and stored in accordance with the guidelines described in the *Handbook for Evaluating Water Bacteriological Laboratories* (20). Either sterile polymethylpentene or glass flasks containing sodium thiosulfate were used to collect the samples.

AC P-A method. The AC P-A test format was either a 100-ml 10-tube most probable number test (1 tube positive denoting the presence of total coliforms in that sample) or a single vessel containing sufficient reagent to receive 100 ml of sample (Access Medical Systems, Branford, Conn.). The powdered formula was manufactured according to previously described specifications (19). Representative samples of both types of P-A tests were subject to quality control procedures described previously (19).

The AC P-A method was performed as follows. For the 10-tube method, 10 ml of water sample was added to each tube, and for the single-vessel method, 100 ml of water sample was added. In both cases the reagent powder was dissolved by agitation, producing a colorless solution. The test tubes or vessels were incubated at 35°C for 24 h. Development of yellow during incubation denoted the presence of total coliforms in either the test tube or the P-A vessel. Each positive total coliform test tube or vessel was exposed to a hand-held fluorescent (366-nm) light (Edmund Scientific Co., Barrington, N.J.). Fluorescence specifically demonstrated the presence of *E. coli*.

Other P-A tests. The revised rules to the Safe Drinking Water Act include the P-A concept, which allows any of three coliform methods to be considered an acceptable P-A test (16). These P-A tests included a 10-tube MTF, with one confirmed tube being a positive result; a MF, with one confirmed sheen colony being considered a positive result; and a 100-ml single fermentation tube (FT) (1).

HPC. A heterotrophic plate count (HPC) was determined for each water sample by using R2A agar incubated at 35°C for 72 h (1).

Evaluation protocol. The comparison of the AC and the three SM P-A tests followed Environmental Support and Monitoring Laboratory guidelines (10). Sufficient water was collected from each location to perform simultaneous P-A tests by the AC and SM procedures. Table 2 shows the distribution of AC and SM P-A analyses performed. Each water sample was divided between a SM P-A and an AC P-A test. All positive presumptive SM tubes or sheen colonies were confirmed as total coliforms by SM procedures (20), and only these were included in the data base. To ensure that a positive test was the result of the target microbes, subcultures were made from both positive SM and AC tests, and bacteria were identified to species by the API 20E system (Analytab Products, Plainview, N.Y.) (12).

The statistics sections of the Department of Epidemiology of Yale University and the Environmental Support and Monitoring Laboratory analyzed the data. Because the data were in the hit-miss (i.e., P-A) mode, comparisons between SM and AC were made in the chi-square form. The Pearson chi-square test of association was used first. Also used were the Mantel-Haenzel test for linear association between rows and columns; the McNemar statistic, which tests whether the disagreements between methods are randomly distributed about the main diagonal; the index of agreement, i.e., the proportion of all the trials for which there are agreement (both presence or both absence); and the kappa statistic,

TABLE 2. National field evaluation of AC and SM tests: P-A comparison

Site	Total no. of samples	No. of each test type			No. of SM[a] tests		No. of AC tests (yellow)		Comparison of P-A results			
		MF	MTF	100-ml vessel	Positive	Negative	Positive	Negative	SM+ and AC+	SM+ and AC−	SM− and AC+	SM− and AC−
California-American Water	196	0	165	31	33	163	41	155	30	3	11	152
SCCRWA	68	68	0	0	20	48	18	50	18	2	0	48
North Andover	92	92	0	0	87	5	80	12	75	12	5	0
Cincinnati	41	41	0	0	13	28	13	28	13	0	0	28
West Penn Water	26	26	0	0	26	0	26	0	26	0	0	0
Washington State	151	0	43	108	21	130	21	130	18	3	3	127
New York City	128	128	0	0	122	6	125	3	122	0	3	3

[a] Composite of all three SM techniques.

which is a "chance-corrected" adjustment to the index of agreement. Kappa ranges from −1 to +1; +1 indicates perfect agreement, 0 indicates no agreement, and negative values suggest less agreement than expected due to chance. The z statistic was calculated for kappa; if this statistic is large (>2), the hypothesis that there was no agreement beyond that expected due to chance is rejected (2, 10, 18).

RESULTS

Comparison of methods. A total of 702 split drinking water samples were analyzed by both methods. Of these, 322 were positive by SM and 324 were positive by AC. Conversely, 380 were negative by SM and 378 were negative by AC (Table 2). The data were further divided into those samples in which both methods were positive (SM+ and AC+), those in which both were negative (SM− and AC−), those in which SM was positive and AC was negative (SM+ and AC−), and those in which SM was negative and AC was positive (SM− and AC+). Both procedures were simultaneously P-A positive in 302 instances, and both were simultaneously negative in 358 samples. The overall agreement rate between the two methods was 94%. SM was positive with a companion AC negative in 20 cases, and SM was negative with an AC positive in 22 cases.

Statistical analyses. The chi-square statistics were generally large. By the Pearson chi-square test, none of the individual locations showed a statistically significant difference in detection rate between the two methods. The chi-square of 2.30 at North Andover was the highest, but it still demonstrated a $P > 0.10$ (Table 3). The Mantel-Haenzel chi-square test showed that the hypothesis of zero correlation was correct. Like the related Pearson chi-square test, it did not show any statistically significant differences overall or at the individual sites. The McNemar chi-square test was used to compare the overall detection rate of positive samples between the two methods. The McNemar test was done for each site to compare the overall proportion of positive samples detected by the two methods. It compared the false-positive and false-negative rates between the two methods. There were no statistically significant differences between SM and AC at any of the individual sites (Table 3).

Kappa was used to measure inter-rate agreement between SM and AC. Kappa values conditional on the SM result were also calculated. For example, kappa$^+$ (conditional) measured agreement between the two methods for only those samples with a positive SM result. Kappa$^-$ (conditional) is similarly interpreted for those samples with a negative SM result. Table 3 presents kappa values and kappa values conditional on the SM results calculated to measure the degree of agreement between the two methods beyond chance agreement. For all but one site, overall agreement was excellent. The results for North Andover indicated chance agreement only. Kappa values conditional on the results of the SM P-A showed that for the California-American site, agreement between the two methods was excellent when SM detected a positive sample but only moderate when the SM result was negative. Conversely, the results for the South Central Connecticut Regional Water Authority site (SCCRWA) were opposite, with agreement in

TABLE 3. Statistical analysis of P-A comparisons

Site	Statistical test values						
	Pearson		McNemar		Kappa		
	Chi-square	P value	Chi-square	P value	Overall (SE)	Conditional	
						Kappa$^+$	Kappa$^-$
California-American Water	0.82	0.37	3.50	0.06	0.77 (0.07)	0.89	0.68
SCCRWA	0.04	0.85	0.50	0.48	0.93 (0.12)	0.86	1.0
North Andover	2.30	0.13	2.12	0.14	−0.08 (0.09)	−0.15	−0.06
Cincinnati	0.06	0.81	0.00	1.00	1.0 (0.16)	1.0	1.0
West Penn Water	0.02	0.89	0.00	1.00	—[a]	—	—
Washington State	0.03	0.87	0.09	0.72	0.83 (0.08)	0.83	0.83
New York City	0.17	0.64	0.03	0.83	0.70 (0.18)	1.0	0.67

[a] —, Not done.

TABLE 4. Species identifications from P-A versus P-A nonagreements

Test result	Site	SM	Species isolated	HPC/ml[a]	Total coliform 100 ml[-1]
SM−/AC+	California-American Water	MTF	*Enterobacter agglomerans*	21,000	7
		MTF	*Citrobacter freundii*	12,000	2
		MTF	*Enterobacter cloacae* *Morganella morgani*	13,000	1
		MTF	*Citrobacter freundii*	17,000	1
		MTF		8,600	23.0
		MTF	*Citrobacter freundii*	24,000	>23.0
		MTF	*Enterobacter amnigenus*	45,000	2
		MTF	*Klebsiella pneumoniae*	210	2
		P-A	*Citrobacter freundii*	1,100	+
		P-A	*Enterobacter agglomerans*	757,000	+
		P-A	*Enterobacter aerogenes*	6,800	+
	SCCRWA	MF	*Enterobacter cloacae*	44	+
	North Andover	MF	*Klebsiella pneumoniae*	N/A	2
		MF	*Enterobacter cloacae*	9	1
		MF	*Klebsiella pneumoniae*	N/A	1
		MF	*Klebsiella pneumoniae*	TNTC	2
		MF	*Klebsiella pneumoniae*	45,000	1
	Washington State	MTF	*Citrobacter freundii*	2,040	1
		MTF	*Serratia fonticola*	310	5
		MTF	*Citrobacter diversus* *Enterobacter agglomerans*	345	2
	New York City	MF	*Escherichia coli*	134	1
		MF	*Enterobacter agglomerans*	5	23.0
			Citrobacter freundii	46	23.0
			Klebsiella pneumoniae		
SM+/AC−	California-American Water	MTF	*Citrobacter freundii*	90	1
		MTF	*Enterobacter aerogenes*	28,000	5
		P-A	*Enterobacter agglomerans*	1,000	+
	SCCRWA	MF	*Klebsiella pneumoniae*	66	1
		MF	*Enterobacter cloacae* *Serratia* sp.	66	1
	North Andover	MF	*Klebsiella pneumoniae*	67,800	3
		MF	*Klebsiella pneumoniae*	42,600	2
		MF	*Klebsiella pneumoniae*	125,000	3
		MF	*Klebsiella pneumoniae*	55,000	3
		MF	*Klebsiella pneumoniae*	7,400	2
		MF		550	3
		MF	*Klebsiella pneumoniae*	235	7
		MF	*Aeromonas* sp.	383,500	2
		MF	*Klebsiella oxytoca*	10,000	7
		MF	*Klebsiella pneumoniae*	53,000	1
		MF	*Klebsiella oxytoca*	42,300	2
		MF	*Klebsiella pneumoniae*	23,000	4
	Washington State	MTF	N/A	N/A	+
		MTF	*Klebsiella pneumoniae*	ca. 11,400	+
		MTF	*Klebsiella pneumoniae*	N/A	+

[a] NA, Not available; TNTC, too numerous to count.
[b] +, Positive result; number of total coliforms not determined.

each case excellent, but stronger when SM was negative. All other sites demonstrated complete agreement.

Characteristics of disagreements. Table 4 shows the bacterial species identifications, HPCs, and total coliforms per 100 ml when SM and AC disagreed. In each case except one, a total coliform was isolated from the AC test. Neither the form of P-A test used nor the HPC exerted any statistically significant effect on the AC results.

Of the disagreements between SM and AC, 90% occurred when the total coliform count per 100 ml was less than 10/100 ml. The majority of these disagreements probably represented the maldistribution of bacteria within the split sample, resulting in sampling error.

A subculture was made from each of the positive AC P-A tests to yield pure cultures and isolates which could be identified to species level to determine if a total coliform was present. Total coliforms were isolated from >98% of positive AC tests (Table 5). *Klebsiella pneumoniae* was the most commonly isolated species, followed by *Enterobacter agglomerans* and *Citrobacter freundii*. *E. coli* was recovered from each fluorescent AC test. Positive AC P-A vessels demonstrated only minimal growth of heterotrophs on subculture. If a large number of heterotrophic bacteria are present in a water sample, all may not die; therefore, when an AC test is subcultured, it is necessary to examine several colonies to ensure that a total coliform is not overlooked.

DISCUSSION

The P-A concept is different in several major respects from the traditional quantitative water analyses. A P-A

TABLE 5. Species of total coliforms isolated[a]

Species	% of all isolates by:	
	SM	AC
Klebsiella pneumoniae	27	30
Klebsiella oxytoca	7	9
Enterobacter agglomerans	11	7
Enterobacter species	2	3
Enterobacter cloacae	19	24
Enterobacter aerogenes	1	1
Citrobacter freundii	14	17
Citrobacter diversus	1	0
Serratia fonticola	2	3
Serratia rubidaea	1	1
Serratia odorifera	1	0
Hafnia alvei	1	0
Escherichia coli	1	1
CDC groups	6	2
Unidentified *Enterobacteriaceae*	6	2

[a] All isolates confirmed in brilliant green lactose broth.

method does not quantify the number of coliform bacteria in a sample but only determines their presence or absence (i.e., hit or miss). It uses a statistical principle to define the minimum acceptable percentage of total coliforms isolated in the system over a particular time span (5, 23, 25). It also decreases the loss of recoverable total coliforms resultant from changes in transportation and storage (3, 5, 23, 25).

The Safe Drinking Water Act regulations propose a maximum contaminant level of no more than 5% of 100-ml samples per month containing total coliform (17). The AC test has demonstrated this level of sensitivity in the laboratory (13), in the national evaluation of the AC most-probable-number method (11), and in the current national evaluation of P-A methods. In this study there was no effect on the sensitivity of the AC method due to the presence of heterotrophic bacteria. During the course of the P-A evaluation, HPCs as high as 700,000/ml were detected. More than 25% of the 702 samples contained over 1,000 HPC per ml.

The specificity of the AC test was established by subculturing positive P-A vessels and identifying the bacteria to species (Table 5). Yellow AC tests yielded total coliforms, and fluorescent AC tests yielded *E. coli*. These results were in keeping with the previous national most-probable-number evaluation, in which positive tests also yielded a species of total coliform when yellow or *E. coli* when fluorescent (11). A theoretical concern about the specificity of the AC was the activity of β-galactosidase containing noncoliforms such as *Aeromonas* spp., which can yield false-positives in SM analyses. Unlike *o*-nitrophenyl-β-D-galactopyranoside tests used for species identification, which depend on bacterial inoculations of 10^7/ml and measure passive β-galactosidase, the AC test uses *o*-nitrophenyl-β-D-galactopyranoside as a defined substrate (13). Therefore, unless there are very large numbers of *Aeromonas* spp. (>10^4 to 10^5/ml) present in the initial drinking-water sample, false-positive AC tests have not been found. This number of *Aeromonas* spp. is unlikely to be encountered in drinking-water samples but if found, should be considered a public health threat because of the association of *Aeromonas* spp. with waterborne disease (22). There was also a concern that bacteria other than *E. coli* might exhibit fluorescence. Therefore, tubes which did not produce yellow were exposed to 366-nm light. In no case did these tubes fluoresce. Thus, there were also no false-positive *E. coli* tests encountered during this survey.

The defined-substrate AC method was configured as a P-A test, which is compatible with the proposed Safe Drinking Water Act regulations. The results reported here show this method to be sensitive and specific for the simultaneous detection of total coliforms and *E. coli* in drinking water. Field testing demonstrated that statistics applicable to P-A tests in general can be used with it. It may be less costly than currently available methods (17).

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