IDEXX
Literature Cover Sheet

IDEXX #: 3A

Title: Comparing Defined Substrate Tests for the Detection of E.coli in Water

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Topic: EPA Study of Chlorine Stressed E. coli.

Highlights: Colilert was compared to EC+MUG for the detection of stressed E. coli in spiked water samples. A total of 33 samples from 27 sources were analyzed. The sources included drinking water spiked with E. coli, effluent from wastewater plants, feces and source water.

Statistical analyses of the data indicated no significant differences in detection of E. coli between Colilert and EC+MUG. The study concluded that Colilert was equivalent to EC+MUG in detecting chlorine-stressed E. coli using both a pure culture and natural populations of E. coli and were capable of detecting 1 cfu/100 mL.

Comparing Defined-Substrate Coliform Tests for the Detection of Escherichia coli in Water

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Two commercially available defined-substrate coliform tests were compared with EC medium plus 4-methylumbelliferyl-β-D-glucuronide (MUG) for detecting chlorine-exposed *Escherichia coli* in spiked water samples. Statistical analyses of the test results indicated no significant differences in detection of *E. coli* between the Autoanalysis Colilert test and EC medium with MUG. There were, however, significant differences in detection of *E. coli* between the ColiQuik test and EC medium with MUG in the free-chlorine-exposed pure culture studies and when all the data were combined. All methods were capable of detecting 1 cfu/100 mL of *E. coli*.

The total coliform group of organisms is the principal indicator used to assess the microbiological quality of drinking water. The sanitary significance of coliform organisms and the characteristics of their culture have been studied extensively.[1-5] The presence of any member of the coliform group in treated water suggests either contamination after disinfection or inadequate treatment. Members of the coliform group are considered a reliable indicator of the adequacy of treatment, but their presence does not necessarily indicate fecal contamination or pathogen occurrence. This shortcoming may be due partly to poor detection of stressed coliforms and to interference by heterotrophs.[6-11] Coliforms, fecal coliforms, and *Escherichia coli* are all used as indicators of fecal pollution. Among these, *E. coli* is often preferred as an indicator because it indicates recent fecal contamination and the possibility of enteric pathogens because enteric pathogens often coexist with fecal coliforms or *E. coli*. The presence of *E. coli* is indicative of fecal contamination. The other members of the coliform group (*Klebsiella, Citrobacter, Enterobacter*) may be isolated in feces, but their presence does not always suggest fecal contamination.

The US Environmental Protection Agency (USEPA) recently amended the National Primary Drinking Water Regulations (NPDWRs),[12] incorporating the maximum contaminant level (MCL), monitoring requirements, and analytical requirements for total coliform bacteria, including fecal coliforms and *E. coli*. The USEPA also promulgated an MCL goal of zero for total coliforms, including fecal coliforms and *E. coli*. The total coliform group remains the primary bacterial indicator. However, for each total coliform-positive sample, a fecal coliform or *E. coli* analysis must be performed. The NPDWRs published June 1, 1990,[13] proposed three analytical methods based on β-glucuronidase (GUR) activity for detecting *E. coli* in drinking water. One of these methods was the minimal medium *o*-nitrophenyl-β-D-galactopyranoside-4-ethylumbelliferyl-β-D-glucuronide (MMO-MUG) or AC* test previously approved for detecting total coliforms in the revised total coliform rule published June 29, 1989.

The presence of any member of the coliform group in treated water suggests either contamination after disinfection or inadequate treatment.

The USEPA approved two of the methods previously proposed for *E. coli* detection in the NPDWRs of Jan. 8, 1991,[14] but deferred approval of the MMO-MUG test because of concerns about its ability to detect low densities of injured *E. coli*.

Several studies have shown that the AC test is comparable to the *Standard Methods* total coliform membrane filter (MF) test, multiple tube fermentation (MTF) test and presence-absence (P-A) coliform test in detecting total coliforms.[14-18] However, there have been only limited studies evaluating the AC test and other similar MUG-based test procedures, e.g., CK,† for detecting *E. coli* in disinfected distribution water.

Thus far two commercially available *o*-nitrophenyl-β-D-galactopyranoside (ONPG)-MUG formulations—AC and CK—appear to be the most prevalent in the marketplace; however, others are rapidly being developed. Both test sys-

*Autoanalysis Colilert, Access Analytical, Branford, Conn.
†ColiQuik, Hach Co., Loveland, Colo.

tems can be used as either a most probable number (MPN) or P–A test.

The AC and CK coliform tests are based on the ability of total coliforms to produce the enzyme β-galactosidase, which hydrolyzes and cleaves the specific substrate ONPG, releasing *o*-nitrophenyl, which produces a yellow color. In addition, the enzyme GUR produced by *E. coli* forms a fluorescent substance when it hydrolyzes MUG. This combination of substrates allows detection of both coliforms and *E. coli* within 24 h. The use of GUR activity to identify *E. coli* was first described by Kilian and Bulow.[19] The association between GUR and *E. coli* has been used to identify this species in a variety of environmental, clinical, and food sources.[15,16,20,21]

The study compared the AC and CK coliform tests with the MTF test using lauryl tryptose broth with MUG (LTB–MUG*)[22] as the presumptive medium and EC medium with MUG (EC–MUG*)[23] for detection of *E. coli*.

Materials and methods

Samples. A total of 33 samples from 27 sources was analyzed (Tables 1 and 2). Aliquots from six of the samples were held for 24 h and for five days after disinfection prior to analyses. Because of difficulties in locating a sufficient number of public drinking water supply systems with ambient levels of *E. coli*, it was necessary to spike oxidant-free treated drinking water (OFTDW) with *E. coli*-positive sources. Sources of *E. coli* included a MUG-positive *E. coli* pure culture, untreated source water, human feces, and primary waste treatment plant effluent. Another reason for using these sources of *E. coli* was to assure that after disinfection there were sufficient *E. coli* cells to detect. The samples of public drinking water supply systems analyzed in this study were *E. coli*–positive, i.e., no spiking was required.

The thermotolerant (EC-positive), MUG-positive *E. coli* used for spiking were isolated from the environment and identified.† The *E. coli* were inoculated into heart infusion broth* and incubated for 24 h at 35°C. The culture was washed three times with OFTDW to remove nutrients from the cells. The cells were resuspended with OFTDW and held for 48 h at 20°C to simulate low-nutrient stress. The suspension was further diluted in OFTDW prior to disinfection.

Feces samples were used as a high-density *E. coli* source by blending approximately 1 g of feces with 200 mL of OFTDW in a sterile blender for 1 min at high speed. The feces suspension was further diluted with OFTDW and was then filtered through sterile filters‡ to remove large particles, lower the turbidity, and lessen the chlorine demand. The suspension was stored at 5°C for 24 h prior to disinfection.

Primary effluents were collected from waste treatment plants that receive primarily domestic influent. Samples were collected aseptically in 4-L sterile polycarbonate sample bottles and returned to the laboratory within 2 h of collection. The effluent samples were filtered similarly to the fecal samples and kept 24 h at 5°C prior to disinfection. *E. coli*–positive public drinking water samples that received no disinfection were collected aseptically in 2-L sterile polycarbonate sample bottles, maintained at 5°C, and analyzed within 48 h of collection.

Disinfection of samples. Microbiological and chemical analyses were performed on the OFTDW used for preparation and dilution of samples. The microbiological analyses included the MF total coliform test[24] and heterotrophic plate counts (HPC) using the spread plate procedure[24] to assure no total coliforms were present prior to spiking and to estimate the HPC levels. Chemical analyses included metals analyses, turbidity, pH, total hardness, alkalinity, sulfate, and nitrate-nitrogen; all were performed according to *Standard Methods*.[24] The OFTDW received all conventional drinking water treatment with the exception of disinfection.

The inactivation experiments were conducted in a similar manner for both the low-nutrient-acclimated pure culture and the fecal suspensions. The respective inocula were added to a beaker containing 400 mL of OFTDW. The initial and final *E. coli* titers after disinfection for calculation of the log reduction were determined by the spread plate procedure using MacConkey agar,* the MF procedure using M-Endo LES agar,*[24] or the MF procedure using M-TEC agar.*[25]

All experiments were conducted at ambient temperature (20–22°C) and pH

*Difco Laboratories, Detroit, Mich.
†API 20E system, Analytab Products, Plainview, N.Y.
‡Whatman No. 40, Maidstone, U.K.

TABLE 1
Comparison of EC-MUG, AC, and CK for detecting E. coli*—pure culture studies*

Sample Number	Disinfectant	Log Reduction	Holding Time	Dilution *mL*	EC-MUG	AC	CK	MPN* E. coli/ *100 mL*	MPN* E. coli/ *tube*
1	Free chlorine 0.52 mg/L† 1 min‡	3.6	0	0.1	8/10	2/10	1/10	1610	1.6
2	Free chlorine 0.40 mg/L 2 min	4.5	0	0.1	10/10	9/10	5/10	>2300	>2.3
3	Free chlorine 0.29 mg/L 4 min	4.5	0	0.1	10/10	9/10	9/10	>2300	>2.3
4	Free chlorine 0.20 mg/L 8 min	5.6	0	1	9/10	9/10	3/10	230	2.3
5	Free chlorine 0.27 mg/L 8 min	6.0	0	0.1	10/10	10/10	6/10	>2300	>2.3
6	Free chlorine 0.60 mg/L 8 min	4.6	24 h	10	10/10	10/10	7/10	>23	2.3
			5 days	1	3/10	4/10	1/10	30	0.3
7	Free chlorine 0.44 mg/L 8 min	5.0	24 h	0.1	4/10	3/10	0/10	5.1	0.51
			5 days	0.1	3/10	1/10	0/10	3.6	0.36
8	Free chlorine 0.42 mg/L 8 min	3.2	24 h	10	3/10	4/10	2/10	3.6	0.36
			5 days	10	2/10	2/10	0/10	2.2	0.22
Total					72/110	63/110	34/110		

*Based on EC–MUG
†Chlorine residual
‡Contact time

TABLE 2

Comparison of EC–MUG, AC, and CK for detecting E. coli*—natural sample studies*

Sample Number	Source	Disinfectant	Log Reduction	Holding Time	Dilution	EC–MUG	AC	CK	MPN* E. coli/ *100 mL*	MPN* E. coli/ *tube*
9	Source water	Free chlorine 0.30 mg/L† 20 s‡	1.2	0	10 mL	10/10	10/10	9/10	>23	>2.3
10	Feces	Free chlorine 0.32 mg/L 40 s	3.7	0	1 mL	1/10	3/10	0/10	10	0.11
11	Feces	Free chlorine 0.40 mg/L 2 min	4.1	24 h	10 mL	2/10	1/10	0/10	2.2	2.2
				5 days	10 mL	4/10	3/10	4/10	5.1	0.51
12	Effluent	Monochloramine 2.8 mg/L 30 min	5.1	24 h	10 mL	3/10	9/10	6/10	3.6	0.36
				5 days	10 mL	9/10	10/10	4/10	23	2.3
13	Effluent	Monochloramine 3.1 mg/L 30 min	5.4	24 h	10 mL	1/10	1/10	4/10	1.1	0.11
				5 days	10 mL	1/10	1/10	0/10	1.1	0.11
14	Drinking water	No disinfectant		48 h	1:6.5	8/10	6/10	6/10	16.1	1.6
15	Drinking water	No disinfectant		48 h	10 mL	10/10	10/10	5/10	>23	>2.3
16	Drinking water	No disinfectant		48 h	10 mL	10/10	8/10	5/10	>23	>2.3
17	Drinking water	No disinfectant		48 h	10 mL	10/10	8/10	7/10	>23	>2.3
18	Effluent	Monochloramine 2.5 mg/L 30 min	5.3	24 h	1 mL	4/10	7/10	0/10	0.51	0.51
19	Effluent	Monochloramine 2.3 mg/L 30 min	5.12	24 h	1 mL	5/10	1/10	0/10	69	0.7
20	Effluent	Monochloramine 3.0 mg/L 30 min	5.42	24 h	1 mL	2/10	3/10	1/10	22	0.22
21	Effluent	Monochloramine 2.7 mg/L 30 min	6.04	24 h	10 mL	0/10	4/10	2/10	<1.1	<0.11
22	Effluent	Monochloramine 2.50 mg/L 30 min	5.70	24 h	10 mL	4/10	3/10	0/10	5.1	0.5
23	Effluent	Monochloramine 2.67 mg/L 30 min	4.84	24 h	1 mL	3/10	4/10	1/10	30.0	0.3
24	Effluent	Monochloramine 2.63 mg/L 30 min	4.80	24 h	10 mL	9/10	9/10	4/10	23.0	2.3
25	Effluent	Monochloramine 2.81 mg/L 30 min	5.10	24 h	1 mL	7/10	7/10	7/10	120	1.2
26	Effluent	Monochloramine 2.76 mg/L 30 min	5.40	24 h	1 mL	1/10	8/10	7/10	110	1.1
27	Effluent	Monochloramine 2.36 mg/L 30 min	5.19	24 h	1 mL	10/10	10/10	8/10	>230	>2.3
Total						114/220	126/220	80/220		

*Based on EC–MUG
†Chlorine residual
‡Contact time

(7.4–8.0), employing a free chlorine residual. The contents of the beakers were continuously stirred (~150 rpm) during the course of the experiment using a multiple stirring device equipped with stainless-steel paddles.* Free chlorine residuals were obtained by the addition of a stock chlorine solution (~1 mg/mL) prepared from a reagent-grade sodium hypochlorite solution.† Free and monochloramine chlorine concentrations were determined by the N,N-diethyl-*p*-phenylenediamine (DPD) colorimetric procedure.[24] Disinfectant levels were measured immediately after the addition of the stock chlorine solution and immediately prior to the end of the exposure time. The action of the oxidant was neutralized by adding 0.5 mL of a stock sterile 10 percent (w/v) sodium thiosulfate solution (aq). Control beakers consisted of the same inoculated OFTDW without oxidant. The controls were treated in the same manner as the test beakers.

The effluents that were used in the inactivation experiments were not diluted with the OFTDW. All experiments utilizing effluents were conducted with combined chlorine residuals (monochloramine). In all other respects, these experiments were conducted in the same manner as the ones conducted with pure culture and fecal inocula, incorporating the appropriate controls.

In the first set of experiments, the leve of inactivation was determined imme diately at the end of the exposure tim (samples 1–5, 9, and 10). Subsequentl the neutralized samples were held at temperature of 5°C for 24 h and for fiv days prior to assay (samples 6–8, 11 13). These holding periods were don to ascertain the true titer present in th inactivated sample for purposes of late dilutions to low levels of *E. coli* and t determine the effect of holding in th absence of a disinfectant residual c the surviving *E. coli* population.

*Phipps and Bird Inc., Richmond, Va.
†Fisher Scientific Co., Pittsburgh, Pa.

TABLE 2

Comparison of EC-MUG, AC, and CK for detecting E. coli*—natural sample studies*

Sample Number	Source	Disinfectant	Log Reduction	Holding Time	Dilution	EC-MUG	AC	CK	MPN* E. coli/ *100 mL*	MPN* E. coli/ *tube*
9	Source water	Free chlorine 0.30 mg/L† 20 s‡	1.2	0	10 mL	10/10	10/10	9/10	>23	>2.3
10	Feces	Free chlorine 0.32 mg/L 40 s	3.7	0	1 mL	1/10	3/10	0/10	10	0.11
11	Feces	Free chlorine 0.40 mg/L 2 min	4.1	24 h	10 mL	2/10	1/10	0/10	2.2	2.2
				5 days	10 mL	4/10	3/10	4/10	5.1	0.51
12	Effluent	Monochloramine 2.8 mg/L 30 min	5.1	24 h	10 mL	3/10	9/10	6/10	3.6	0.36
				5 days	10 mL	9/10	10/10	4/10	23	2.3
13	Effluent	Monochloramine 3.1 mg/L 30 min	5.4	24 h	10 mL	1/10	1/10	4/10	1.1	0.11
				5 days	10 mL	1/10	1/10	0/10	1.1	0.11
14	Drinking water	No disinfectant		48 h	1:6.5	8/10	6/10	6/10	16.1	1.6
15	Drinking water	No disinfectant		48 h	10 mL	10/10	10/10	5/10	>23	>2.3
16	Drinking water	No disinfectant		48 h	10 mL	10/10	8/10	5/10	>23	>2.3
17	Drinking water	No disinfectant		48 h	10 mL	10/10	8/10	7/10	>23	>2.3
18	Effluent	Monochloramine 2.5 mg/L 30 min	5.3	24 h	1 mL	4/10	7/10	0/10	0.51	0.51
19	Effluent	Monochloramine 2.3 mg/L 30 min	5.12	24 h	1 mL	5/10	1/10	0/10	69	0.7
20	Effluent	Monochloramine 3.0 mg/L 30 min	5.42	24 h	1 mL	2/10	3/10	1/10	22	0.22
21	Effluent	Monochloramine 2.7 mg/L 30 min	6.04	24 h	10 mL	0/10	4/10	2/10	<1.1	<0.11
22	Effluent	Monochloramine 2.50 mg/L 30 min	5.70	24 h	10 mL	4/10	3/10	0/10	5.1	0.5
23	Effluent	Monochloramine 2.67 mg/L 30 min	4.84	24 h	1 mL	3/10	4/10	1/10	30.0	0.3
24	Effluent	Monochloramine 2.63 mg/L 30 min	4.80	24 h	10 mL	9/10	9/10	4/10	23.0	2.3
25	Effluent	Monochloramine 2.81 mg/L 30 min	5.10	24 h	1 mL	7/10	7/10	7/10	120	1.2
26	Effluent	Monochloramine 2.76 mg/L 30 min	5.40	24 h	1 mL	1/10	8/10	7/10	110	1.1
27	Effluent	Monochloramine 2.36 mg/L 30 min	5.19	24 h	1 mL	10/10	10/10	8/10	>230	>2.3
Total						114/220	126/220	80/220		

*Based on EC-MUG
†Chlorine residual
‡Contact time

(7.4–8.0), employing a free chlorine residual. The contents of the beakers were continuously stirred (~150 rpm) during the course of the experiment using a multiple stirring device equipped with stainless-steel paddles.* Free chlorine residuals were obtained by the addition of a stock chlorine solution (~1 mg/mL) prepared from a reagent-grade sodium hypochlorite solution.† Free and monochloramine chlorine concentrations were determined by the N,N-diethyl-*p*-phenylenediamine (DPD) colorimetric procedure.[24] Disinfectant levels were measured immediately after the addition of the stock chlorine solution and immediately prior to the end of the exposure time. The action of the oxidant was neutralized by adding 0.5 mL of a stock sterile 10 percent (w/v) sodium thiosulfate solution (aq). Control beakers consisted of the same inoculated OFTDW without oxidant. The controls were treated in the same manner as the test beakers.

The effluents that were used in the inactivation experiments were not diluted with the OFTDW. All experiments utilizing effluents were conducted with combined chlorine residuals (monochloramine). In all other respects, these experiments were conducted in the same manner as the ones conducted with pure culture and fecal inocula, incorporating the appropriate controls.

In the first set of experiments, the level of inactivation was determined immediately at the end of the exposure time (samples 1–5, 9, and 10). Subsequently, the neutralized samples were held at a temperature of 5°C for 24 h and for five days prior to assay (samples 6–8, 11–13). These holding periods were done to ascertain the true titer present in the inactivated sample for purposes of later dilutions to low levels of *E. coli* and to determine the effect of holding in the absence of a disinfectant residual on the surviving *E. coli* population.

*Phipps and Bird Inc., Richmond, Va.
†Fisher Scientific Co., Pittsburgh, Pa.

AC and CK coliform tests. AC and CK tubes containing sufficient defined substrate for 10 mL of sample were prepared as a 10-tube MPN test. A sample (10, 1.0, or 0.1 mL) was added to each tube, and the powder was dissolved with agitation. To AC and CK tubes that received 1 or 0.1 mL of sample, 9 mL or 9.9 mL, respectively, of sterile water or buffer was added, consistent with the manufacturers' instructions.

The MPN AC and CK tubes were incubated at 35 ± 0.5°C for 24 ± 0.5 h. Each tube was exposed to a hand-held long-wavelength (366-nm) 6-W UV light.* Fluorescence indicated the presence of *E. coli* (MUG test). Doubtful MUG-positive tubes were incubated for an additional 4 h and were also compared with a color comparator to assess any degree of fluorescence so as to not underestimate the *E. coli* density. A positive control (*E. coli*) was included with each sample.

LTB–MUG and EC–MUG tests. The 10-tube MTF test was performed by adding 10, 1, or 0.1 mL of sample to LTB–MUG tubes. The tubes were incubated at 35 ± 0.5°C, and positive tubes showing gas or heavy growth within 24 or 48 ± 0.5 h were read for fluorescence and transferred to EC–MUG with sterile hardwood applicator sticks. The EC-MUG tubes were incubated at 44.5 ± 0.2C in a gable-covered water bath for 24 ± 0.5 h. All LTB-MUG and EC-MUG tubes were exposed to a hand-held long-wavelength (366-nm) UV light. Fluorescence indicated the presence of *E. coli* (MUG test).

MUG-negative tubes. All LTB–MUG-, EC–MUG-, AC-, and CK-MUG-negative tubes (no fluorescence) were membrane filtered† according to *Standard Methods* using a modification of the M-TEC method for *E. coli*. LTB–MUG-negative tubes were filtered in the pure culture studies, and EC–MUG-negative tubes were filtered with the natural sample comparison studies. LTB–MUG-negative tubes were filtered in the pure culture studies because none of the EC–MUG tubes failed to show a positive MUG response upon transfer from the LTB–MUG tubes. One- and 9-mL portions of each MUG-negative tube were filtered, and the MF was placed in petri dishes (50 × 9 mm) containing 5 mL of plate count agar‡ and incubated for 2 h at 35 ± 0.5°C to allow chlorine-exposed organisms a chance to repair. The MFs were then placed on petri dishes (50 × 9 mm) containing 5 mL of M-TEC agar‡ and incubated for 22 h at 44.5 ± 0.2°C in sealed plastic bags§ in a gable-covered circulating water bath. Presumptive *E. coli* colonies (yellow colonies) on the MFs were streaked for isolation on MacConkey agar and incubated at 35°C for 24 ± 0.5 h. The isolates were reinoculated into LTB–MUG tubes. MFs with confluent growth were rubbed with a sterile swab, a small portion of the surface of MacConkey agar plates was rubbed and subsequently streaked for isolation with a sterile loop, and LTB–MUG tubes were inoculated. The tubes were incubated at 35 ± 0.5°C, and positive tubes showing gas or heavy growth within 24 or 48 ± 0.5 h were transferred to EC–MUG with sterile hardwood applicator sticks. The EC–MUG tubes were incubated at 44.5 ± 0.2°C for 24 ± 0.5 h. All LTB–MUG and EC–MUG tubes were exposed to a hand-held long-wavelength (366-nm) UV light. Fluorescence indicated the presence of *E. coli* (MUG test). Isolates from samples that were spiked with natural sources were further identified as *E. coli*.**[26]

TABLE 3
*Percentage of false negatives**

Source	Medium		
	EC–MUG	AC	CK
Pure culture	2.6	6.4	32.9
Natural sample	16.4	23.4	18.6
All samples	10.7	14.9	26.0

*Percentage of MUG-negative tubes by each method for which isolates of these tubes were MUG-positive when reinoculated into LTB–MUG and EC–MUG

Statistical analyses. The *E. coli* recoveries by the EC–MUG, CK, and AC tests were evaluated by the Wilcoxon signed rank test.[27] The Wilcoxon signed rank test was performed by arranging the N differences between pairs in order of size, ignoring their signs. Rank numbers were then assigned to these absolute differences, rank 1 being given to the smallest difference, rank 2 to the next smallest, etc., and rank N to the largest. The signs of the original differences were then restored to the rank numbers, and T_+, the sum of the positive rank numbers, is the test statistic. In the event that ties occur among the differences, the same procedure as in the rank sum test is used. The tied differences are each given the average rank numbers that would have been assigned had the differences not been tied. The hypothesis tested was that there is no difference in detection rates by the two methods. The data collected in this study were segregated into three elements for statistical analyses—comparison of the three methods (EC–MUG, AC, and CK) using a pure culture of *E. coli*, comparison using naturally occurring *E. coli*, and an overall comparison of all data. Critical values for the signed rank test were obtained from tables in Wilcoxon et al.[28] All statistical tests were performed at an alpha level of 0.05.

Results

Comparison of LTB–MUG, EC–MUG, AC, and CK. A total of 438 tubes of LTB-MUG were positive (turbidity or gas or both), and 301 tubes of EC–MUG were MUG-positive. A total of 313 tubes were MUG-positive using the AC test, 220 tubes were MUG-positive using the CK test, and 278 tubes of LTB–MUG were MUG-positive.

Aliquots (10-, 1-, and 0.1-mL sample portions) were inoculated into LTB–MUG in an effort to get a span of positive and negative tubes for evaluation purposes. Ten-millilitre sample portions of the public drinking water samples were examined in accordance with the total coliform rule. The compilation of data presented in Tables 1 and 2 reflects only the results of 10 tube tests for which there was a breakpoint, i.e., positive and negative tubes.

Tables 1 and 2 show the number of MUG-positive tubes by each method, the disinfectant results, and the estimate of the *E. coli* density after disinfection for all samples. Table 1 shows the results of the pure culture studies, and Table 2 shows the results of the natural sample comparison studies. A total of 186 EC tubes, 189 AC tubes, and 114 CK tubes were MUG-positive. A total of 168 tubes were MUG-positive using LTB–MUG. The LTB–MUG data are not shown in Tables 1 and 2. The agreement of AC and CK with the EC–MUG method was 102 and 61.3 percent, respectively. *E. coli* was detected in all samples using the AC test; however, *E. coli* was not detected in one of the 33 samples using EC–MUG and nine of the 33 samples using the CK coliform test with the same sample volumes. The log reduction after disinfection ranged from 1.2 to 6.04. The density of *E. coli* in the disinfected samples ranged from 1.1 to >2,300 *E. coli* organisms/100 mL. The *E. coli* density per tube ranged from <0.11 to >23.

Statistical analyses. The Wilcoxon signed rank test was applied to all samples; the pure culture and natural sample studies indicated no significant difference ($P > 0.05$) in the number of positive tubes or detection of *E. coli* between the EC–MUG test and the AC test. Statistical analyses of the data comparing CK with EC–MUG for detection of *E. coli* showed a significant difference ($P < 0.05$), with the EC–MUG method resulting in more positive tubes across all samples and

*UVP Inc., San Gabriel, Calif.
†HA, 0.45-um pore size, Millipore Corp., Bedford, Mass.
‡Difco Laboratories, Detroit, Mich.
§Whirl-pak, Nasco, Ft. Atkinson, Wis.
**API 20E system, Analytab Products, Plainview, N.Y.

with the pure culture studies. However, there were no statistically significant differences in detection of *E. coli* between EC–MUG and CK with the natural samples. The EC–MUG method yielded more positive tubes than the AC test in 12 samples, whereas the AC test yielded more positive tubes in 10 of the samples. Both methods resulted in equal numbers of positive tubes in 10 samples. The EC–MUG method yielded more positive tubes than the CK coliform test in 28 samples, whereas the CK method resulted in more positive tubes in only four of the samples. Both EC–MUG and CK resulted in the same number of positive tubes in two of the samples.

False-negative tubes. Table 3 shows the percentage of MUG-negative tubes by each method in which isolates of these tubes were MUG-positive when reinoculated into LTB–MUG and EC–MUG (false negative). With the exception of CK (32.9), the lowest false-negative rates were observed with EC–MUG (2.6) and AC (6.4) using a free-chlorine-exposed pure culture of *E. coli*. The false-negative rates with natural populations of *E. coli* ranged from 16.4 percent with EC-MUG, followed by 18.6 percent with the CK test to 23.4 percent using the AC test. The lowest percentage of false-negative tubes overall was with EC–MUG (10.7), followed by the AC test (14.9) and CK (26.0). There were instances in which isolates from MUG-negative tubes were MUG-positive with EC–MUG, but *E. coli* was not isolated from MacConkey agar plates. This was because, in many cases, there was confluent growth on the MFs from filtration of the MUG-negative tubes, and *E. coli* was detected by swabbing the surface of the MF and transferring that to LTB–MUG. However, because of the limited number of colonies picked for identification, *E. coli* colonies were undoubtedly present but were sometimes missed. This was not a frequent occurrence, i.e., less than 20 percent of these isolates were not identified as *E. coli*. The predominant background organism was *Klebsiella pneumoniae*. The number of MUG-positive tubes given in Tables 1 and 2 was not corrected for the false-negative results.

Undetected target error. Table 4 shows the percentage error introduced in specificity resulting from undetected *E. coli* calculated by ASTM standard D 3870-79.29 The closer each calculated value is to zero percent, the more specific the method. The lowest calculated value, i.e., best specificity, was with EC–MUG (7.8 percent) for all samples, followed by AC (9.7 percent) and CK (37.2 percent). The lowest value (1.3 percent) was with EC–MUG using pure cultures of free-chlorine-exposed *E. coli*. Generally, the percentages were higher in the natural sample studies compared with the pure culture studies, with the exception of the CK coliform test.

Effect of holding disinfected samples. Table 5 presents the results of holding studies. Six of the samples were held 24 h and 5 days after disinfection to determine the effects of holding in the absence of disinfectant residual on the surviving *E. coli* population. With the possible exception of sample 6, which was indeterminate (i.e., MPN >23), the remaining samples showed no significant changes in *E. coli* density within the five-day holding period.

Characteristics of OFTDW. Table 6 shows the characteristics of the OFTDW. The values for the analytes were below the MCLs of the primary and secondary drinking water regulations,[30] with the exception of turbidity, which exceeded the MCL of 0.5 ntu.

Discussion

Both the AC and CK tests are novel departures from classical total coliform cultural methods that depend on lactose fermentation to detect the presence of coliforms. The AC and CK coliform tests use the substrate ONPG (for total coliforms) and MUG (for *E. coli*) both for essential nutrients and as the indicator system (yellow color and fluorescence). The tests are designed so that no additional confirmation tests are needed. Positive ONPG tubes are relatively easy to read. A positive MUG test using AC is easy to detect because of the brilliantly fluorescing tubes; however, the MUG reaction is sometimes difficult to interpret with CK, LTB–MUG, and EC–MUG tubes showing heavy growth.

Statistical analyses using the Wilcoxon signed rank test show that there was no significant statistical difference ($P>0.05$) between the EC–MUG method and AC for detecting *E. coli*. There was a significant difference ($P<0.05$) between EC–MUG and the CK coliform test, with the EC–MUG method showing better detection of free-chlorine-exposed *E. coli*. The results of this study do not agree completely with the findings of other similar CK and AC evaluation studies. Ziel and Mick[31] found AC and CK comparable to LTB–MUG for detection of *E. coli* in spiked distribution samples. McCarty et al[32] found CK and AC equivalent to LTB–MUG for the recovery of *E. coli* from spiked disinfected distribution samples. Clark et al[33] showed that there was a significant difference between the MFC method, the AC test, and the CK test for detecting *E. coli* in treated water samples, with the MFC method being more sensitive. Gale and Broberg[34] found in their evaluation of AC that the minerals-modified glutamate MTF test was significantly better in detecting and enumerating *E. coli* in both untreated and chlorinated water samples. They used the same statistical test used in this study. Differences in the outcomes of

TABLE 4
*Percentage of undetected target errors**

Source	Medium		
	EC-MUG	AC	CK
Pure culture	1.3	4.5	42.3
Natural sample	16.6	16.4	30.2
All samples	7.8	9.7	37.2

*Calculated by ASTM Standard D 3870-79

TABLE 5
Effect of holding disinfected samples

Sample Number	Holding Time	MPN* E. coli/*100 mL*
6	24 h	>23
	5 days	30
7	24 h	5.1
	5 days	3.6
8	24 h	3.6
	5 days	2.2
11	24 h	2.2
	5 days	5.1
12	24 h	3.6
	5 days	23
13	24 h	1.1
	5 days	1.1

*Based on EC-MUG

these studies may be attributed to different sample types, different media comparison combinations, and different forms of stress to the organisms.

The results of this study corroborate those of Edberg and Edberg,[35] who were able to detect 1 cfu/100 mL of chlorine-exposed *E. coli* with a MUG-based substrate. All samples in this study, with the exception of the drinking water samples, received chlorine disinfection. Using the AC test, *E. coli* was detected in all samples. It was detected in 32 of the 33 samples using EC–MUG and in 24 of the samples using the CK coliform test. After disinfection, the mean *E. coli* count was 23 cfu/100 mL. The mean was somewhat skewed by the relatively high levels of *E. coli* in the first several samples of the study. The median *E. coli* count was 22 cfu/100 mL. The mean number of *E. coli* organisms per tube was 1.2, and the median was 0.9.

Six of the disinfected samples were held five days after disinfection (Table 5) to assess any repair that may have occurred, as evidenced by significant changes in *E. coli* density. Any differences observed between the *E. coli* levels at 24 h and at five days after disinfection were not significant. The 24-h and five-day MPN values for each sample were within the 95 percent confidence limits.

However, the laboratory-simulated nutritional and disinfection stress may not closely approximate the stress applied to *E. coli* in treated drinking water. These data do not completely agree with other studies that examined the effects of holding time and temperature on the survival of coliforms. McDaniels and Bordner[36] examined the survival of total coliforms in municipal drinking water distribution system samples held at both ambient temperature (22°C) and 5°C. Coliform populations declined significantly at both temperatures after 24 h. Average losses in 24 h were 34 percent at 5°C and 87 percent at 22°C. However, it is not known whether these samples contained *E. coli*. McFeters et al[37] reported survival times for *E. coli* of one to five days in well water, and Flint[38] reported survival times of up to 260 days at temperatures from 4 to 25°C for *E. coli* introduced into filter-sterilized river water. Many other holding time studies with total coliforms or *E. coli* have been reported, but basic differences in the conditions of the studies make comparisons difficult.

A major factor in the disparity of the results of the studies comparing EC-MUG to the other tests was the occurrence of false-negative tubes. Increasing the incubation time from 24 to 28 h did not result in significant changes in the number of MUG-positive tubes by any of the methods. This is similar to the results of Clark et al.[33] Sixteen percent of the isolates from MUG-negative EC–MUG, 23 percent of the isolates from AC MUG-negative tubes, and 19 percent of the isolates from CK MUG-negative tubes were MUG-positive upon transfer to LTB–MUG and EC–MUG, suggesting that exposure to halogen disinfection may result in the inability of the organisms to utilize the MUG substrate. This was again demonstrated in the pure culture studies in which a known MUG-positive *E. coli* isolate was exposed to chlorine and, in some cases, was MUG-negative for all the MUG methods, but when it was reinoculated into LTB–MUG and EC–MUG, it was MUG-positive. This was particularly true with the CK coliform test, which showed a relatively high false-negative rate with this particular strain of *E. coli* or which may be less efficient in general for detecting free-chlorine-exposed *E. coli*. These observations support the conclusions of McFeters[11] that coliforms in water systems may be undetected because sublethal stress leads to decreased detection on conventional media.

Feng et al[39] reported that the glucuronidase gene may be present in non-MUG-utilizing strains of *E. coli* but is not expressed. In the research of Bej et al,[40] the glucuronidase gene was amplified by the polymerase chain reaction (PCR) before hybridization with a DNA probe. The DNA probe confirmed that the glucuronidase gene was present in all *E. coli*, including *E. coli* 0157:H7 strains, as well as in some shigellae. The authors concluded that glucuronidase activity is under some form of catabolite repression in MUG-negative strains of *E. coli*. Kasper et al[41] reported that antibodies to glucuronidase reacted with extracts of three of four MUG-negative strains. These results show that in some strains of *E. coli*, glucuronidase is produced but is inactive, the substrate does not enter some strains, or the 4-methylumbelliferyl is not released.

Studies that have documented the incidence of false-negative results for MMO–MUG-based substrates are limited. Clark et al[33] reported false-negative occurrences of 12 and 19 percent with CK and AC, respectively, with untreated waters and 61 and 81 percent (CK and AC, respectively) with treated water samples positive for *E. coli*. Covert et al[18] reported the percentage of false negatives in their evaluation of the AC test for total coliforms to be 20.5 percent; however, there were too few *E. coli*-positive samples to evaluate the efficacy of the AC test for detecting *E. coli*. In this study, the false-negative rates for EC–MUG, CK, and AC using chlorine-exposed natural populations of *E. coli* were 16.4, 18.6, and 23.4, percent respectively. Using ASTM standard practice D 3870-79 for establishing the performance characteristics of microbiological methods, the percentages of undetected target errors using chlorine-exposed natural populations of *E. coli*, the AC test and EC–MUG were similar.

TABLE 6
Characteristics of oxidant-free treated drinking water

Parameter	Units	Analytical Value
Turbidity	ntu	1.3
Chloride	mg/L	19.5
Sulfate	mg/L	90
Nitrate-N	mg/L	1.0
Sodium	mg/L	13.7
Calcium	mg/L	39.9
Magnesium	mg/L	10.6
Hardness as $CaCO_3$	mg/L	150
Alkalinity as $CaCO_3$	mg/L	67.6
pH	pH units	8.05
Copper	mg/L	<0.02
Manganese	mg/L	<0.05
Lead	mg/L	<0.002
Iron	mg/L	<0.04
Zinc	mg/L	<0.01
Specific conductance	micromhos at 25°C	410
Total coliforms	cfu/100 mL	<1
Mean heterotrophic plate count	cfu/mL	1,700

Summary

Statistical analyses of the data indicated no significant difference in detection of *E. coli* between the AC test and EC–MUG; however, there were statistically significant differences between the CK coliform test and EC–MUG using a free-chlorine-exposed pure culture of *E. coli* and when the data for all samples were combined. The AC test was equivalent to EC–MUG in detecting free-chlorine-exposed *E. coli* using a pure culture and monochloramine-exposed natural populations of *E. coli*. There were no statistically significant differences in detection of *E. coli* with CK using monochloramine-exposed natural populations of *E. coli*. All the methods evaluated were capable of detecting 1 cfu/100 mL of *E. coli*. The lowest false-negative rate or undetected target error was with EC–MUG. In view of the lack of published studies addressing false-negative occurrences or

rates, more definitive studies are needed to establish the false-negative rates with MUG-based methods using chlorine-exposed environmental populations of *E. coli*. Also, the observation that *E. coli* posed to halogen disinfection may metimes be unable to utilize MUG substrate warrants additional study.

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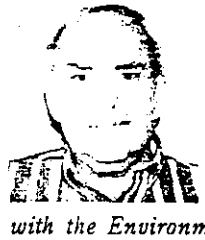
References

1. CLARK, H.F. & KABLER, P.W. Reevaluation of the Significance of the Coliform Bacteria. *Jour. AWWA*, 56:7:931 (July 1964).
2. EVANS, T.M. ET AL. Coliform Species Recovered From Untreated Surface Water and Drinking Waters by Membrane Filter, Standard and Modified Most-Probable Number Techniques. *Appl. Envir. Microbiol.*, 41:657 (1981).
3. GELDREICH, E.E. ET AL. Type Distribution of Coliform Bacteria in the Feces of Warm-Blooded Animals. *Jour. WPCF*, 34:2295 (1962).
4. KABLER, P.W. & CLARK, H.F. Coliform Group and Fecal Coliform Organisms as Indicators of Pollution in Drinking Waters. *Jour. AWWA*, 52:12:1577 (Dec. 1960).
5. KABLER, P.W.; CLARK, H.F.; & GELDREICH, E.E. Sanitary Significance of Coliform and Fecal Coliform Organisms in Surface Water. *Public Health Repts.*, 79:58 (1964).
6. EVANS, T.M. ET AL. Failure of the Most-Probable-Number Technique to Detect Coliforms in Drinking Water and Raw Water Supplies. *Appl. Envir. Microbiol.*, 41:130 (1981).
7. LIN, S. Evaluation of Coliform Tests for Chlorinated Secondary Effluents. *Jour. WPCF*, 45:498 (1973).
8. OLSON, B.H. Enhanced Accuracy of Coliform Testing in Seawater by a Modification of the Most-Probable-Number Method. *Appl. Envir. Microbiol.*, 36:438 (1978).
9. ALLEN, M.J. & GELDREICH, E.E. Bacteriological Criteria for Ground-Water Quality. *Ground Water*, 13:1 (1975).
10. GELDREICH, E.E. ET AL. The Necessity of Controlling Bacterial Populations in Potable Water: Community Water Supply. *Jour. AWWA*, 64:9:596 (Sept. 1972).
11. MCFETERS, G.A. Enumeration, Occurrence, and Significance of Injured Indicator Bacteria in Drinking Water. *Drinking Water Microbiology* (G.A. McFeters, editor). Springer-Verlag, New York (1990).
12. National Primary Drinking Water Regulations: Total Coliforms (including fecal coliforms and *E. coli*). *Fed. Reg.*, 54:27544 (June 29, 1989).
13. National Primary Drinking Water Regulations: Analytical Techniques; Coliform Bacteria; Proposed Rule. *Fed. Reg.*, 55:22752 (June 1, 1990).
    National Primary Drinking Water Regulations: Analytical Techniques; Coliform Bacteria. *Fed. Reg.*, 56:636 (Jan. 8, 1991).
15. EDBERG, S.C.; ALLEN, M.J.; & SMITH, D.B. 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. *Appl. Envir. Microbiol.*, 54:1595 (1988).
16. EDBERG, S.C.; ALLEN, M.J.; & SMITH, D.B. 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. *Appl. Envir. Microbiol.*, 55:1003 (1989).
17. LEWIS, C.M. & MAK, J.L. Comparison of Membrane Filtration and Autoanalysis Colilert Presence–Absence Techniques for Analysis of Total Coliforms and *Escherichia coli* in Drinking Water Samples. *Appl. Envir. Microbiol.*, 55:3091 (1989).
18. COVERT, T.C. ET AL. Evaluation of the Autoanalysis Colilert Test for Detection and Enumeration of Total Coliforms. *Appl. Envir. Microbiol.*, 55:2443 (1989).
19. KILIAN, M. & BULOW, P. Rapid Diagnosis of Enterobacteriaceae. I. Detection of Bacterial Glycosidases. *Acta. Pathol. Microbiol. Scand. Sect. B*, 84:245 (1976).
20. HARTMAN, P.A. The MUG (Glucuronidase) Test for *Escherichia coli* in Food and Water. *Rapid Methods and Automation in Microbiology and Immunology* (A. Bolows, R.C. Tilton, and A. Turano, editors). Tipografia Queriniava, Bresica, Italy (1989).
21. ROBISON, B.J. Evaluation of a Fluorogenic Assay for Detection of *Escherichia coli* in Foods. *Appl. Envir. Microbiol.*, 48:285 (1984).
22. FENG, P. & HARTMAN, P.A. Fluorogenic Assays for Immediate Confirmation of *Escherichia coli*. *Appl. Envir. Microbiol.*, 43:1320 (1982).
23. RIPPEY, S.; CHANDLER, L.; & WATKINS, W. Fluorometric Method for Enumeration of *Escherichia coli* in Molluscan Shellfish. *Jour. Food Protection*, 50:685 (1987).
24. *Standard Methods for the Examination of Water and Wastewater*. APHA, AWWA, and WPCF. Washington, D.C. (17th ed., 1989).
25. DUFOUR, A.P.; STRICKLAND, E.; & CABELLI, V.J. Membrane Filter Method for Enumeration of *Escherichia coli*. *Appl. Envir. Microbiol.*, 41:1152 (1981).
26. EDBERG, S.C. ET AL. Clinical Evaluation of the MICRO-ID, API 20E, and Conventional Media Systems for Identification of Enterobacteriaceae. *Jour. Clin. Microbiol.*, 10:161 (1979).
27. WILCOXON, F.A. & WILCOX, R.A. Some Rapid Approximate Statistical Procedures. American Cyanimid Co., New York (1964).
28. WILCOXON, F.; KATTI, S.; & WILCOX, R.A. Critical Values and Probability Levels for the Wilcoxon Rank Sum Test and the Wilcoxon Signed Rank Test. American Cyanimid Co., New York (1963).
29. Standard Practice for Establishing Performance Characteristics for Counting Methods in Bacteriology. ASTM D 3870-79 (1979).
30. National Secondary Drinking Water Regulations. Final Rule. *Fed. Reg.*, 44:42195 (July 19, 1979).
31. ZIEL, C.A. & MICK, T.A. A New Enzymatic Substrate Test for the Detection of Total Coliforms and *Escherichia coli*. Proc. 1990 AWWA WQTC, San Diego, Calif.
32. MCCARTY, S.C. ET AL. Defined Substrate Technology and Polymerase Chain Reaction–Gene Probe for the Detection of Total Coliforms and *Escherichia coli* in Water. Proc. 1990 AWWA WQTC, San Diego, Calif..
33. CLARK, D.L. ET AL. Comparative Study of Commercial 4-Methylumbelliferyl-β-Glucuronide Preparations With the Standard Methods Membrane Filtration Fecal Coliform Test for the Detection of *Escherichia coli* in Water Samples. *Appl. Envir. Microbiol.*, 57:1528 (1991).
34. GALE, P. & BROBERG, P. Evaluation of the Colilert Technique. FRO 132. Foundation for Water Research, Bucks, U.K. (1990).
35. EDBERG, S.C. & EDBERG, M.M. A Defined Substrate Technology for the Enumeration of Microbial Indicators of Environmental Pollution. *Yale Jour. Biol. Med.*, 61:389 (1988).
36. MCDANIELS, A.E. & BORDNER, R.H. Effects of Holding Time and Temperature on Coliform Numbers in Drinking Water. *Jour. AWWA*, 75:9:458 (Sept. 1983).
37. MCFETERS, G.A. ET AL. Comparative Survival of Indicator Bacteria and Enteric Pathogens in Well Water. *Appl. Microbiol.*, 27:823 (1974).
38. FLINT, K.P. The Long-Term Survival of *Escherichia coli* in River Water. *Jour. Appl. Bacteriol.*, 63:261 (1987).
39. FENG, P.; LUM, R.; & CHANG, G. Presence of β-D-Glucuronidase Gene Sequences in MUG Assay (-) *Escherichia coli*. Abstracts 1990 Ann. Mtg. Amer. Soc. Microbiol., Anaheim, Calif.
40. BEJ, A.K. ET AL. Detection of *Escherichia coli* Based Upon Polymerase Chain Reaction and Amplification of Uid. Abstracts 1990 Ann. Mtg. Amer. Soc. Microbiol., Anaheim, Calif.
41. KASPAR, C.W.; HARTMAN, P.A.; & BENSON, A.K. Coagglutination and Enzyme Capture Tests for the Detection of Escherichia coli, Galactosidase, β-Glucuronidase and Glutamate Decarboxylase. *Appl. Envir. Microbiol.*, 53:1073 (1987).



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