Enumeration of Total Coliforms and *Escherichia coli* from Source Water by the Defined Substrate Technology

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Many water utilities are required to monitor source water for the presence of total coliforms, fecal coliforms, or both. The Colilert system, an application of the defined substrate technology, simultaneously detects the presence of both total coliforms and *Escherichia coli* directly from a water sample. After incubation, the formula becomes yellow if total coliforms are present and fluorescent at 366 nm if *E. coli* is in the same sample. No confirmatory tests are required. The Colilert system was previously assessed with distribution water in a national evaluation in both most-probable-number and presence-absence formats and found to produce data equivalent to those obtained by using *Standard Methods for the Examination of Water and Wastewater* (*Standard Methods*). The Colilert system was now compared with *Standard Methods* multiple-tube fermentation (MTF) for the enumeration of total coliforms and *E. coli* from surface water. All MTF tubes were confirmed according to *Standard Methods*, and subcultures were made to identify isolates to the species level. Colilert tubes were subcultured to determine if color changes were specific to the target microbes. The Colilert system was found equally sensitive to MTF testing by regression, *t* test, chi-square, and likelihood fraction analyses. Specificity of the Colilert system was shown by the isolation of a species of total coliform or *E. coli* after the appropriate color change. The Colilert test can be used for source water samples when enumeration is required, and the benefits previously described for distribution water testing—sensitivity, specificity, less labor, lower cost, faster results, no noncoliform heterotroph interference—are applicable to this type of water analysis.

The defined substrate technology, applied to water analysis as the Colilert system, can simultaneously detect and enumerate total coliforms and *Escherichia coli* directly from water samples. The method is easy to perform. All ingredients are in powder form in test tubes for the quantitative most-probable-number (MPN) method and in containers for presence-absence (P-A) analysis. A measured amount of water is added to each tube or container, and the powder is dissolved. A colorless solution results. The tubes are placed in a 35°C incubator for 24 h. The test tubes that contain total coliforms become yellow. The yellow tubes are then exposed to a hand-held fluorescent light (4 W, 366 nm). Tubes with *E. coli* will fluoresce brightly. No confirmatory or completed tests are required.

A national evaluation of the Colilert system to determine equivalency to U.S. Environmental Protection Agency-approved *Standard Methods for the Examination of Water and Wastewater* (*Standard Methods*) was sponsored by the Environmental Protection Agency and the American Water Works Association Research Foundation. Distribution system water from 10 geographical areas representing a broad range of sources, both surface and subterranean, was tested. The Colilert system was compared with *Standard Methods* in both the MPN (quantitative) (7) and P-A (qualitative) (8) modes. In both the quantitative and qualitative modes, there was no statistical difference between *Standard Methods* and the Colilert system. Species identifications from positive tubes confirmed the sensitivity of the Colilert system. The comparison showed that the Colilert system was as sensitive as *Standard Methods* MPN and P-A, specifically enumerated one total coliform per 100 ml, simultaneously enumerated one *E. coli* per 100 ml in the same analysis, was not

subject to false-positive or false-negative results by noncoliform heterotrophic bacteria, did not require confirmatory tests, was easy to inoculate, and was very easy to interpret (7, 8).

Because many utilities analyze source water in addition to distribution water, a comparison between *Standard Methods* and the Colilert system for this purpose was undertaken. Split samples of raw water surface sources were analyzed for total coliforms and *E. coli* by the multiple-tube fermentation method and the Colilert system.

MATERIALS AND METHODS

Samples. Water samples were taken from surface source systems of the Bridgeport Hydraulic Company (Bridgeport, Conn.) and the South Central Connecticut Regional Water Authority (New Haven, Conn.). Together, these utilities serve a population of approximately 900,000 in an area of 1,500 mi² (ca. 3,883 km²). Water samples were collected, transported, and stored in strict accordance with the guidelines described by *Standard Methods* (1). Sterile polymethylpentene or glass flasks were used to collect the samples (2, 4). Source water was diluted with sterile, dechlorinated tap water to result in a final total-coliform count in the range of 1 to 20 total coliforms per 100 ml so that meaningful statistical comparisons could be made. All comparative analyses were performed with split samples from the same water or origin (6).

Defined substrate technology. The Colilert system was used with 100-ml samples (Access Analytical Systems, Branford, Conn.). It was formatted in a 10-tube MPN arrangement. The water samples were added to the Colilert tubes. Each tube contained enough Colilert powder to receive 10 ml of water. The contents of the tubes were shaken to dissolve the powdered formula. A colorless solution resulted. The ves-

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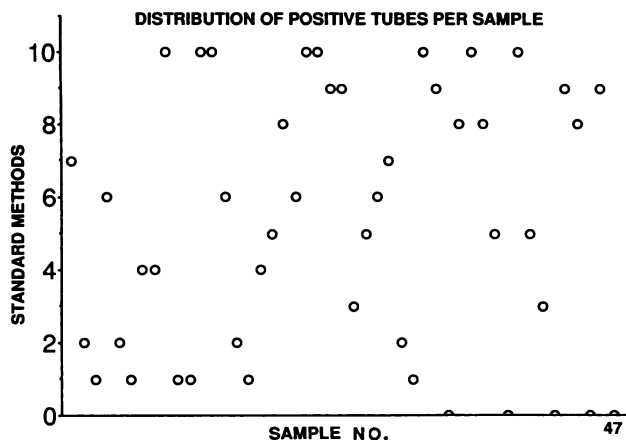
DISTRIBUTION OF POSITIVE TUBES PER SAMPLE

FIG. 1. The distribution of MPN values per test for each of 47 tests, as seen in the sample population during the study. Numbers are tubes positive by *Standard Methods* analysis.

TABLE 1. Species of total coliforms isolated

Species	% of all isolates identified by:	
	Standard Methods	Colilert system
Klebsiella pneumoniae	24	27
Klebsiella oxytoca	13	12
Enterobacter agglomerans	10	7
Enterobacter species	4	3
Enterobacter cloacae	19	22
Enterobacter aerogenes	1	3
Citrobacter freundii	9	13
Citrobacter diversus	1	1
Serratia fonticola	6	5
Serratia rubidaea	1	2
Serràtia odorifera	1	0
Hafnia alvei	1	0
Escherichia coli	6	1
Centers for Disease Control groups	2	2
Unidentified *Enterobacteriaceae*	2	2

sels were then placed in a 35°C (±2°C) incubator for 24 hours.

A yellow color in the vessel after incubation denoted the presence of total coliforms. Any positive total-coliform test tube was exposed to a hand-held fluorescent (366 nm) light. Fluorescence in the test tube indicated the presence of *E. coli*. Therefore, a separate result was obtained for both total coliforms and *E. coli*. The number of coliforms per 100 ml was estimated from a 10-tube MPN table (1). No confirmatory or completed tests needed to be performed. At least one positive Colilert test tube from each positive water sample was subcultured, and the colonies were identified by species by the API 20E system (Analytab Products, Plainview, N.Y.), with supplementary tests as necessary (10).

Multiple-tube fermentation test. The multiple-tube fermentation test was performed as a 10-tube MPN test, with each tube containing 10 ml of double-strength lactose tryptose broth (Difco Laboratories, Detroit, Mich.) (1). Positive tubes were confirmed in brilliant green bile lactose broth (Difco) (1). The number of coliforms per 100 ml was estimated from a 10-tube MPN table. Only confirmed lactose tryptose broth tubes were included in the data base for comparison with the Colilert system.

Heterotrophic-plate-count bacteria. Noncoliform heterotrophic-plate-count bacteria were determined for each water sample with R2A agar (Difco) incubated at 35°C for 48 h (1).

RESULTS

A total of 47 split samples were analyzed. The distribution of positive tubes was wide, varying from 0 of 10 to 10 of 10 (Fig. 1).

The *Standard Methods* multiple-tube fermentation test showed a mean for all samples of 5.25 positive tubes with a standard deviation of 3.59. A total of 247 tubes were positive. The Colilert system showed a mean of 5.36 positive tubes per samples with a standard deviation of 3.3. The total number of tubes positive by the Colilert system was 252. Species of total coliforms isolated by both methods are listed in Table 1.

Although the number of positive tubes by each method agreed closely, there was considerable variation around the mean (Fig. 2). The correlation coefficient r^2 was 0.514, indicating modest correlation between the two methods (Fig.

2). The disparity between the close means and the wide range of correlations has two likely explanations. First, very small numbers of microbes were divided into two separate volumes (i.e., split samples). The maximum number of bacteria analyzed in this study was 16/100 ml, or less than 0.2/ml. A relatively wide distribution range of these low bacterial densities between the two split samples would be expected (17). Further compounding the potential for maldistribution is the fact that microbes in water tend to be associated with particles. The particles would be of uneven size, would have unequal numbers of bacteria on them, and could also be subject to uneven distribution (18, 19). Second, many of the samples were stored overnight in a refrigerator before being processed. This was often required in order to perform a screening test to determine how many bacteria
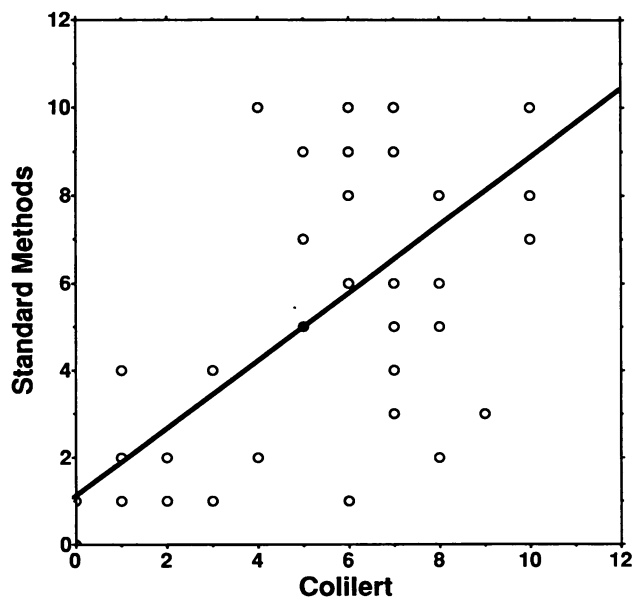

FIG. 2. Comparison of Colilert system and *Standard Methods* analysis by regression analysis. The range around the line of regression is much greater than that seen with distribution water. $y = 0.7736x + 1.1074$; $r^2 = 0.5136$. Numbers are positive tubes per test.

were in the sample and thus ascertain the amount of sterile water diluent to add. The overnight storage at 4°C would exacerbate maldistribution by increasing the formation of particle precipitation.

When the results obtained by the two methods were statistically compared, the Wilcoxon signed-rank showed 16 ranks with a negative sign, for a total sum rank of 275.5 and mean rank of 17.2; in addition, it showed 16 positive ranks, for a total sum rank of 252.5 and mean rank of 15.8. Fifteen cases were eliminated for differences of zero. The Z value was -0.215 with a P value of 0.83. The Z value corrected for ties was also -0.215 with the same P value. There was no significant difference between the two methods according to this chi-square test. Likewise, the Spearman correction coefficient demonstrated a sum D^2 of 5,940, Rho of 0.6566, and Z of 4.45. The rho corrected for ties was 0.65. Here also there was no statistical difference between the two methods.

An additional measure of possible differences between the two methods is the t test. This test measures differences along the line of regression and determines if any disparities exist. Although the t test is most often employed for comparing results from chemical analysis, it is used by the Environmental Protection Agency to examine biological data (6). The mean x-y was 0.11, and the paired t value was 0.28. The probability of the two-tailed paired t test was 0.78, indicating no statistically significant difference between the methods (2, 13).

The likelihood ratio test compares the estimates of mean bacterial density obtained from different sets of data (16). The calculation of the likelihood ratio (16) from the individual likelihood function L (16) by the formula showed the two methods yielded equivalent results at the -2 ln significant at 0.5%.

It is known that Standard Methods tests can be suppressed by noncoliform heterotrophs (14, 15). Although the Colilert system has not been shown to be so affected by distribution water, an analysis with source water was conducted here to address this question. There was no effect on the Colilert system positivity on the basis of heterotrophic-plate-count bacterial density (Fig. 3). The r^2 value was 0.07, with an adjusted r^2 value of 0.05. The analysis of variance yielded an F test of 3.55 with a P value of 0.07. The confidence interval table showed mean (x,y) values for lower limits of 4.2 for 95% and 4.3 for 90% and upper limits of 5.0 for 95% and 4.9 for 90%. The slopes were -0.008 for the 95% lower limit, 0.01 for the 90% lower limit, 0.23 for the 95% upper limit, and 0.21 for the 90% upper limit.

DISCUSSION

The Colilert system has previously been compared with Standard Methods procedures in both the MPN (7) and P-A formats (8). It was examined with distribution water from a wide variety of sources, including well, ground, surface, and river. In the MPN format, the Colilert system produced results that were equivalent to those obtained with the multiple-tube fermentation MPN, with an r^2 of 0.779 (7). The likelihood ratio test showed no significant difference, with a likelihood ratio of 0.5% (16). The precision of the Colilert system was greater than that of Standard Methods, with both F and Scheffe tests demonstrating statistically significant increases. In addition, the Colilert system was refractory to noncoliform heterotrophic bacteria interference, whereas the Standard Methods procedure showed definite suppression in a number of cases (14, 15). In the P-A format there was 94% agreement between the Colilert system and
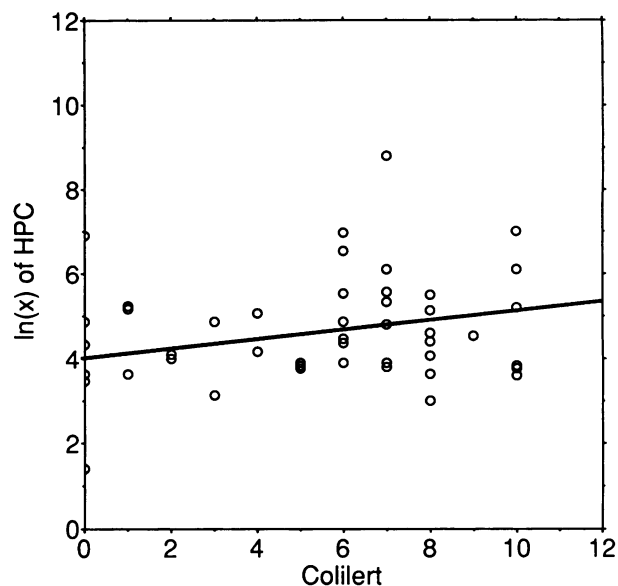

FIG. 3. Effect of concentrations of noncoliform heterotrophs on the reactivity of the Colilert system. Noncoliform heterotrophs did not demonstrate suppression. $y = 0.1098x + 4.0$; $r^2 = 0.0731$. Numbers are positive tubes per test.

Standard Methods. All chi-square analyses demonstrated no statistically significant differences between the two methods (8). The U.S. Environmental Protection Agency found a 92% agreement with positive tubes and a 98% agreement overall (6a).

On the basis of these studies and investigations undertaken by the Environmental Protection Agency, the Colilert system has been proposed as an alternative test procedure for the analysis of total coliforms from drinking water. In addition, its ability to provide a simultaneous *E. coli* determination makes it an extremely useful public health tool and highly compatible with new proposed Safe Drinking Water Act regulations (12).

Many states require utilities to monitor source water in addition to distribution water. Several differences between source and distribution water could have an effect on analytic techniques. Source water, especially surface water, has a wider spectrum and greater number of microbes than distribution water. There is more opportunity to experience a false-positive test because of synergistic gas or acid production (3) or high numbers of *Aeromonas* species, which may produce reactions in the lactose tryptose or brilliant green bile lactose broths, which make them indistinguishable from total coliforms (14) in these procedures. Likewise, the potential for false-negative tests is greater with source water because of the larger and more diverse number of noncoliform heterotrophs present. Heterotrophic-plate-count bacteria counts above 500/ml have been associated with false-negative total coliform tests by both multiple-tube fermentation and MF media (11). Furthermore, chemical differences between source and distribution water may exert an effect. The amount of inorganic chemicals such as corrosion inhibitors and residual chlorine (a form of chlorine), the potential activity of heavy metals from pipes, and the activity of biofilm may all exert effects on the analytic test (3–5, 9, 11, 15, 19).

The defined substrate technology, based on a different principle than fermentation-based media, should not be

affected by the biological variability of source water, and the buffers in it should make it refractory to the chemical differences (7). The broad range of noncoliform heterotrophs would not find the nutrient matrix present in the Colilert system suitable for their growth. Therefore, heterotrophic-plate-count bacteria suppression, which should not be a problem with the Colilert system, was verified here. Also, since the Colilert system does not support the growth of *Aeromonas* species and similar lactose-fermenting non-coliforms, false-positives are minimized.

The examination of source water is required by many states. Utilities often perform both total coliform and fecal coliform analyses routinely. The Colilert system, providing a simultaneous analysis for total coliforms and *E. coli*, can provide utilities a quantitative (MPN) measure of both these indicators simultaneously. Analysts should be aware, however, that because of the heterogeneous distribution of bacteria in a sample, especially from source water, comparative results may vary on a sample-by-sample basis.

Therefore, the defined substrate technology Colilert system offers the accurate, simultaneous analysis of total coliforms and *E. coli*. In addition, the method offers the same benefits—results of a complete analysis in 24 h and ease of performance and interpretation—in analyzing source water as those shown with distribution water.

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