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Literature Cover Sheet

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Title: Evaluation of Colilert-Marine for Detection of Total Coliforms and *Escherichia coli* in Marine Water

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Topic: Use of Colilert in Marine Water

Highlights:

- Equivalent to MTF for the detection of *E. coli*.
 - Provides a simple and rapid method to assess fecal contamination.
 - Useful tool with which to monitor coastal beach water.
- * See highlights page 789, page 789 & page 790.

Evaluation of Colilert-Marine Water for Detection of Total Coliforms and *Escherichia coli* in the Marine Environment

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A test that allows for early detection of fecally contaminated coastal water would enhance public health protection. Colilert-Marine Water (Colilert-MW; Environetics, Branford, Conn.) is a rapid 24-h test that has recently been developed to detect total coliforms and *Escherichia coli* in coastal water. We performed a premarketing evaluation of the Colilert-MW product, testing it in parallel with the multiple tube fermentation (MTF) method for 86 coastal water samples in southern California. Statistical analysis was performed by using paired *t* tests and linear regression. Bacterial isolates were evaluated by biochemical and genetic analysis. The results of this study showed a strong correlation between the traditional MTF and the Colilert-MW method for detection of total coliforms ($r = 0.95$) and *E. coli* ($r = 0.89$) in ocean water samples. Paired *t*-test results indicated that the Colilert-MW and MTF were equivalent in detecting *E. coli* and that the Colilert-MW may be more sensitive in the detection of total coliforms. We conclude that Colilert-MW would be a useful tool with which to monitor coastal beach water.

A new product, Colilert-Marine Water (Colilert-MW), has been developed by Environetics (Branford, Conn.) to simultaneously detect total coliforms and *Escherichia coli* in marine water within 24 h. Similar to the Colilert product developed for fresh water, the new marine water test relies on the substrates *O*-nitrophenyl- β -D-galactopyranoside (ONPG) and 4-methylumbelliferyl- β -D-glucuronide (MUG) to detect total coliforms and *E. coli*, respectively. The presence of coliforms is indicated by a change in the medium from clear to yellow, while the presence of *E. coli* is determined by fluorescence under long-wave (366-nm) UV light. The new formulation has a reduced amount of sodium chloride in the medium in order to accommodate marine water samples.

The freshwater Colilert has been extensively tested. National studies conducted by Edberg et al. (5, 6) tested the Colilert at water utilities in different geographical areas and found that the product accurately detected total coliforms and *E. coli* in drinking water. Rice and coworkers determined that 95.5% of *E. coli* strains from humans, cows, and horses fluoresced in the medium and that the test distinguished *E. coli* from other *Escherichia* species (14, 15). Clark et al. (3) found that the Colilert was equivalent to membrane filtration in the detection of *E. coli* from untreated surface water but that it did not effectively recover *E. coli* from chlorine-treated water samples. More recently, the Colilert method and *E. coli* medium with MUG (ECMUG) were both found to be useful for the detection of chlorine-exposed *E. coli* (4, 12).

The ability of the Colilert to detect total coliforms and *E. coli* in fresh water has been established. The Colilert for fresh water was approved by the U.S. Environmental Protection Agency for total coliforms in 1989 (8) and for detection of *E. coli* in June 1992 (9). These approvals have provided water utilities and public health agencies with the opportunity to quickly and efficiently determine the quality of fresh water. The development of a rapid method for

detecting coliforms in ocean water would provide similar benefits to agencies that monitor the marine environment and provide quick results that could aid in protecting public health. We evaluated the new Colilert-MW product in parallel with standard multiple tube fermentation (MTF) testing to determine the level of agreement and its potential usefulness for ocean coliform monitoring. The presence of total coliforms and *E. coli* was confirmed by using biochemical identification and genetic analysis.

MATERIALS AND METHODS

Sampling. Over a period of several weeks, 86 water samples were collected in sterile bottles from 17 nearshore beach surf zone stations in southern California. All samples were kept on ice and processed within 4 h after collection. Initially, 26 ocean samples were tested in parallel with the MTF monitoring. An additional 60 samples were tested in February 1992, when southern California received heavy rains. These rains contributed to minor sewage overflows and caused storm drain overloads to enter coastal waters, resulting in a 1-mile beach closure in the monitored area. This presented a unique opportunity to test the Colilert-MW under harsh environmental conditions.

MTF. Fifteen-tube MTF tests were performed as described in *Standard Methods* prepared (1). Briefly, 1:10, 1:100, and 1:1,000 dilutions were prepared, inoculated into lauryl tryptose broth (LTB), and incubated at 35°C for 24 to 48 h. During the rainy period, 1:100, 1:1,000, and 1:10,000 dilutions were used for sites that had high coliform counts. A loopful of broth from tubes containing LTB that showed positive results (positive LTB tubes) was transferred to brilliant green bile broth (BGB) and ECMUG. BGB tubes were incubated at 35°C for 24 to 48 h. ECMUG tubes were incubated at 44.5°C for 24 h.

Colilert-MW. Concurrent studies comparing the Colilert-MW with the traditional MTF were completed by rehydrating Colilert-MW tubes with 9 ml of sterile distilled water and 1 ml of coastal water sample. The other Colilert-MW tubes were inoculated from the same dilution tubes that were

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used for the 1:100, 1:1,000, and 1:10,000 dilutions in the MTF procedure. Tubes were incubated at 35°C for 24 h. An additional test was completed for 20 separate samples by using 10 ml of undiluted ocean water to rehydrate the Colilert-MW tubes.

Bacterial analysis. The first 26 samples were subjected to intensive examination. Samples from all 1:10 dilution tubes (plus positive tubes from other dilutions) were streaked onto MacConkey agar plates. In addition, 11 negative and 3 positive ECMUG tubes were streaked onto MacConkey agar plates. Samples showing representative morphologies were streaked for isolation on Trypticase soy agar (TSA). Bacterial isolates were identified biochemically with the API20E (Analytab Products, Plainview, N.Y.).

Since two isolates of *Vibrio cholerae* non-O1 were recovered from one of the first 17 sites tested, thiosulfate-citrate-bile salts-sucrose (TCBS) medium was included during testing of the last 9 sites. Samples from the Colilert-MW tubes that tested positive were streaked onto TCBS (in addition to MacConkey agar) and incubated at 35°C for 24 h. Representative morphologies were identified as described above.

Genetic analysis. The UAR-900 oligonucleotide (5'-AC GCGTGGTTACAGTCTTGCG-3'), described by Bej et al. (2), detects a portion of the *uidA* gene. The *uidA* gene codes for β -glucuronidase, a 1.8-kb single-copy gene responsible for fluorescence in *E. coli* when MUG is metabolized.

Genomic DNA was extracted from bacterial isolates by using the G-NOME extraction kit (Bio 101, Inc., La Jolla, Calif.) and resuspended in TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) buffer. One microgram of extracted DNA was diluted in 100 μ l of TE, heat denatured at 100°C for 10 min, and placed on ice for 5 min. One hundred microliters of 20 \times SSC (1 \times SSC is 150 mM NaCl plus 15 mM sodium citrate [pH 7.0]) was added to each sample. The samples were immediately slot blotted onto Hybond-N⁺ membranes (Amersham, Arlington Heights, Ill.) with a Minifold II filtration apparatus (Schleicher & Schuell, Keene, N.H.). The DNA was cross-linked for 2 min with a Stratalinker 2400 (Stratagene, La Jolla, Calif.).

The oligonucleotide probe was synthesized (Genosys Biotechnologies, Woodlands, Tex.) and labeled by using the Genius 5 kit (Boehringer Mannheim, Indianapolis, Ind.). The membrane was prehybridized for 2 h at 58°C in prehybridization solution (5 \times SSC, 0.1% *N*-lauroylsarcosine, 0.02% sodium dodecyl sulfate [SDS], 2% Boehringer Mannheim blocking reagent) and hybridized with the UAR-900 probe (2 pmol/ml) for 12 h at 58°C. The membranes were washed twice in 2 \times SSC-0.1% SDS at 58°C for 15 min and twice in 0.5 \times SSC-0.1% SDS at 51°C for 15 min. Probe hybridization was detected by using the Genius 3 (Boehringer Mannheim) chemiluminescence kit per the manufacturer's instructions.

E. coli isolates were also screened to determine the presence of any enterohemorrhagic strains. *E. coli* cells that did not fluoresce in Colilert-MW or ECMUG medium were tested for the somatic O157 antigen by using the *E. coli* O157:H7 latex test reagent kit (Pro-Lab Diagnostics, Round Rock, Tex.).

Statistics. Statistical analysis was completed by an independent company, EcoAnalysis (Ojai, Calif.). The results from the Colilert-MW and MTF methods were compared by paired *t* tests. In addition, Pearson correlation coefficients and principal-axis regression slopes were computed to quantify the relationships between the two methods. Principal-axis regression analysis was used instead of the standard least-squares regression tests to compensate for the sampling error associated with both methods. The standard

regression method assumes that there is no error in one of the variables. Since analyses with raw data values were dominated and distorted by a small number of relatively large values, all analyses were performed with values transformed by a natural logarithm.

RESULTS

Comparison of standard MTF and Colilert-MW results for recovery of total coliforms and *E. coli*. Overall results for the total coliform test showed that 605 tubes with BGB were positive and 622 tubes were positive in the Colilert-MW. With ECMUG, which identifies *E. coli*, 249 tubes fluoresced and produced gas while 247 Colilert-MW tubes fluoresced, indicating the presence of *E. coli*.

We compared the most-probable-number values obtained with the Colilert-MW with the most-probable-number values obtained by the traditional MTF test. For purposes of analysis, the data were compared in three different groups. Group 1 included all 86 samples, group 2 included the 66 samples in which the standard dilutions (1:10, 1:100, and 1:1,000) were used, and group 3 included only the high-dilution (1:100, 1:1,000, and 1:10,000) data.

Paired *t*-test analysis of *E. coli* recovery (Table 1) strongly indicated that there was no difference between the two tests for the three groups since none of the *P* values were significant. Results from paired *t* tests for total coliform recovery showed that the Colilert-MW method gave significantly higher most-probable-number values for groups 1 (*P* = 0.02) and 2 (*P* = 0.01). The mean ln differences for groups 1 and 2 indicated that the results from the Colilert-MW were 22 and 27% higher, respectively ($e^{0.20} = 1.22$ and $e^{0.24} = 1.27$). However, the results of the total coliform sample analyses for group 3 were not significant (*P* = 0.75), indicating there was no difference between the Colilert-MW and MTF tests during storm conditions.

The data were also analyzed by standard linear regression analysis. The principal-axis slopes were close to 1.0 for all *E. coli* and total coliform groups. The principal-axis slope confidence limits for all analyses, except for *E. coli* standard dilutions, covered the value of 1.0. Strong overall *r* values for comparison of recovery of total coliforms (*r* = 0.95) and *E. coli* (*r* = 0.89) were obtained.

The facts that groups 1 and 2 of total coliforms had significant *t*-test values and slopes of 1.0 are not a contradiction. These results may be due to the fact that the *y* intercept of the regression equation is less than zero (the *y* intercepts for the two analyses are -0.69 and -0.72, respectively).

Bacterial analysis. Twenty-six (30%) of the 86 water samples were subjected to intensive testing to determine the rate of false-positive and false-negative results with Colilert-MW medium. For the 26 water samples, 390 tubes (15 tube dilutions \times 26 samples) were tested; 83 turned yellow and 41 also fluoresced. In the total coliform portion of the test, a false-positive result was defined as a Colilert-MW tube that turned yellow but contained no coliforms, and a false-negative result was a tube that remained clear but contained coliforms. For the *E. coli* portion of the test, a false-positive result was a Colilert-MW tube that turned yellow and fluoresced, but from which no *E. coli* organisms were recovered. A false-negative result was defined as a tube that turned yellow, contained *E. coli*, but did not fluoresce.

Total coliforms. Coliforms were not recovered from 16 (19%) of the 83 yellow Colilert-MW tubes (Table 2). *Aeromonas hydrophila*, *V. cholerae* non-O1, *Kluyvera* species,

TABLE 1. Comparison of Colilert-MW and MTF results

Organism and group	No. of samples	Mean in difference ^a	t	P^b	r^c	PA slope ^d	95% confidence limits ^e
Total coliform							
1	86	-0.20 (22%)	-2.32	0.02	0.95	1.071	0.998-1.150
2	66	-0.24 (27%)	-2.62	0.01	0.95	1.079	0.995-1.170
3	20	-0.07	-0.32	0.75	0.73	1.048	0.675-1.640
<i>E. coli</i>							
1	86	-0.04	-0.43	0.67	0.89	1.079	0.966-1.199
2	66	0.03	0.28	0.78	0.90	1.192	1.059-1.344
3	20	-0.25	-1.06	0.30	0.63	1.060	0.602-1.903

^a Mean difference between the paired ln-transformed data values (MTF and Colilert). For tests with a significant t value, the percent difference corresponding to the mean in difference is given in parentheses.

^b t value from the paired t test.

^c Type-1 probability associated with the t test.

^d Pearson correlation coefficient.

^e Slope of the principal-axis regression equation.

^f 95% confidence limits of the principal-axis slope.

and 10 organisms that were not identified in the API20E data base were recovered from these tubes. No false-negative results were found. Tubes that remained colorless and did not fluoresce contained *Providencia rettgeri*, *Vibrio alginolyticus*, *Acinetobacter calcoaceticus*, and several organisms that were not identified in the API20E data base.

E. coli. *E. coli* was not recovered from 6 (15%) of the 41 Colilert-MW tubes that turned yellow and fluoresced (Table 2). *Serratia liquefaciens*, *Klebsiella pneumoniae*, and four isolates that were not identified in the API20E data base were recovered from these tubes. The above six isolates were all negative for fluorescence upon reinoculation, suggesting that they were not the cause of the fluorescence during the original test. Five (12%) of 42 yellow Colilert-MW tubes contained *E. coli* but did not fluoresce. *E. coli* isolates from four of these five tubes fluoresced upon reinoculation into Colilert-MW medium.

Eleven negative ECMUG tubes that were tested for accuracy included five that produced gas but did not fluoresce and six that fluoresced but did not produce gas. Organisms recovered from tubes that only produced gas included *Serratia oleronensis*, two *E. coli* isolates, *Serratia* spp., and one organism that was not identified by the API20E. Organisms recovered from ECMUG tubes that fluoresced but did not produce gas included two organisms that were not in the API data base, *Serratia* spp., two *E. coli* isolates, and *Escherichia fergusonii*. In summary, 4 (36%) of the 11 tubes contained *E. coli* isolates but would not have been considered positive. The *E. coli* isolates did not fluoresce or produce gas when reinoculated into ECMUG.

Vibrio spp. Two tests were performed to determine whether *Vibrio* spp., a predominant group in ocean waters, caused interference. In one test, nine Colilert-MW tubes, rehydrated with 9 ml of sterile distilled water and inoculated

with 1 ml of undiluted seawater, were plated on both MacConkey agar and the TCBS medium. *Vibrio* growth on TCBS medium was observed and verified by the API20E. No vibrios were recovered from MacConkey agar plates. Other coliforms, including *E. coli*, were recovered from MacConkey agar plates.

In a separate experiment, 20 Colilert-MW tubes were inoculated with 10 ml of undiluted ocean water. Fourteen (70%) of 20 were yellow and fluoresced. Samples from the positive tubes were streaked in duplicate onto MacConkey plates. Where possible, five isolates per plate were streaked for isolation on TSA and identified by the API20E. Of 91 isolates identified by the API20E, 54 were *Vibrio* spp. (*V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus*, and *V. fluvialis*), 36 isolates were oxidase positive but their profiles were not in the API20E data base, and one isolate was identified as *E. coli*.

Molecular analysis. DNA was extracted from isolates recovered from the first 26 water samples and hybridized with the UAR-900 probe. *E. coli* ATCC 43886 was used as a positive control, and *E. fergusonii* and *Escherichia hermannii*, recovered from other ocean water samples, were used as negative controls. The presence of DNA that hybridized to the UAR-900 probe was detected in 17 of 17 *E. coli* isolates from yellow, fluorescent Colilert-MW tubes and from 4 of 5 *E. coli* isolates (Fig. 1, lane A, 4, 7, and 10, and lane B, 3 and 4) recovered from yellow, nonfluorescent Colilert-MW tubes. One of two *E. coli* isolates from BGB tubes hybridized with the probe (lane B, 9 and 10). No hybridization was detected for *V. fluvialis*, *V. cholerae*, *K. pneumoniae*, *Citrobacter freundii*, or five isolates that were not identified by the API20E.

Four of four *E. coli* isolates recovered from negative ECMUG tubes hybridized with the UAR-900 probe (data not shown). None of the *E. coli* isolates from Colilert-MW and ECMUG tubes agglutinated when tested with the latex O157 antigen detection kit.

TABLE 2. False-positive and false-negative test results

Organism	No. (%) of results:			
	Positive		Negative	
	True	False	True	False
Total coliforms	67 (81)	16 (19)	20 (100)	0 (0)
<i>E. coli</i>	35 (85)	6 (15)	37 (88)	5 (12)

DISCUSSION

Pollution of nearshore coastal environments is a growing concern for public health agencies, regulators, and the general public. Coastal areas can be adversely impacted by a variety of events, including (i) heavy rains that cause storm drain overflow, (ii) agricultural and urban runoff, (iii) rup-

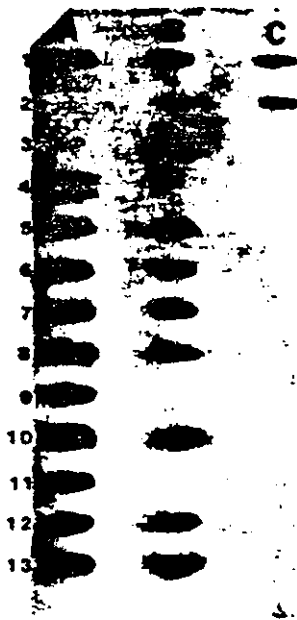


FIG. 1. Hybridization signals of bacterial DNA hybridized with the UAR-900 oligonucleotide probe. A1, *E. coli* ATCC 43886; A2, *E. fergusonii*; A3, *E. hermannii*; A4 to B10, *E. coli*; B11, isolate not identified by API20E; B12 to C2, *E. coli*; C3, *Klebsiella oxytoca*; C4 and C5, *V. cholerae*; C6, *K. pneumoniae*; C6 and C7, *C. freundii*; C8 to C10, isolates not identified by API20E.

es in sewage discharge pipes, and (iv) boats illegally discharging human waste. Many coastal states monitor ocean water by using the standard MTF to detect total or fecal coliforms. The MTF with LTB and BGB media takes up to 4 days to obtain results, is labor intensive, and unless ECMUG is used, does not identify *E. coli*. A rapid and efficient method to test marine water and the development of specific federal regulations requiring all coastal states to monitor beach waters on a regular basis are needed to ensure that beaches are safe for human contact.

This study evaluated a new, rapid, 24-h product proposed for testing marine waters, the Colilert-MW, and compared the results with those of the traditional MTF method. Statistical analysis using paired *t* tests indicated that there was no difference between the two tests for the detection of *E. coli*. For the detection of total coliforms, however, the Colilert-MW gave higher numbers than did the MTF method. Bacterial analysis showed recovery of three species that are known to be ONPG positive, *A. hydrophila*, *V. cholerae*, and *Kluyvera* species, from the Colilert-MW tubes. Thus, depending on the relative abundance of these organisms in a given coastal area, the Colilert-MW test could yield falsely elevated numbers of total coliforms. In an earlier study, Ellgas et al. (7) also reported higher total coliform recovery in the marine environment when the original Colilert was used. They hypothesized that the Colilert medium did a better job of resuscitating coliforms stressed by exposure to highly saline bay water than does conventional recovery medium. This possibility cannot be overlooked.

Bacterial analysis of the first 26 water samples revealed false-positive rates of 19 and 15% for total coliform and *E. coli* detection, respectively. False-positive reactions obtained by the traditional MTF method applied to marine

water have been previously documented (11). Non-*E. coli* isolates recovered from yellow, fluorescent tubes did not fluoresce upon reinoculation, suggesting that they were not the original cause of fluorescence. Additionally, only one loopful was streaked, and it is highly possible that the target coliforms were present in the positive Colilert-MW tubes but were not recovered. Thus, the false-positive rates are not alarming.

Importantly, coliforms were not recovered from Colilert-MW tubes that remained clear. Previous work has shown that the traditional MTF method may have a 50% false-negative rate for ocean water (13). Therefore, the lack of false-negative results with the Colilert-MW product is encouraging.

Biochemical identification studies have shown that 10 to 20% of environmental *E. coli* strains are negative in MUG medium (3, 4, 16). In our study, *E. coli* isolates were identified in 5 of 41 yellow, nonfluorescent Colilert-MW tubes, indicating a MUG-negative rate of 12%. Genetic analysis indicated that four of these *E. coli* isolates contained sequences that hybridized with the UAR-900 probe. This suggests that the *uidA* gene, although present, was not expressed. This phenomenon has also been observed by Feng et al. (10) and Bej et al. (2). Interestingly, false-negative results were found in 4 of 11 ECMUG tubes that showed atypical results, suggesting that the standard recovery medium for *E. coli* may also be inadequate for ocean monitoring.

The number of *Vibrio* isolates is normally higher than the number of coliforms in coastal water. Typically, we recovered 80 to 100 presumptive *Vibrio* organisms per 10 ml of southern California coastal water while the number of coliforms is less than seven per 10 ml. For this reason, we needed to determine how *Vibrio* spp. would react in the Colilert-MW medium. The fact that *Vibrio* species inhibited coliforms in the Colilert-MW medium was apparent when over 50% of the organisms recovered from 90 Colilert-MW tubes that were rehydrated with undiluted seawater were *Vibrio* spp. This was not the case for tubes rehydrated with 9 ml of sterile distilled water and 1 ml of undiluted marine water since coliforms were easily recovered.

Since marine water is the natural habitat for *Vibrio* bacteria, undiluted ocean water provides vibrios with a competitive advantage over the coliform group. Conversely, rehydrating Colilert-MW tubes with sterile fresh water and seawater dilutions may provide coliforms with an advantage in that marine vibrios do not survive in fresh water and may lyse upon entering these tubes. Therefore, it is recommended that the Colilert-MW not be rehydrated with undiluted seawater but that dilutions, starting with 1:10, are made to perform the test.

In summary, the new Colilert-MW product is equivalent to the traditional MTF method for the detection of *E. coli* but not for the detection of total coliforms. The higher recovery of total coliforms may be attributed to increased sensitivity or interference from other ONPG-positive organisms in coastal water. However, testing of coastal water for the presence of total coliforms may not be appropriate. Many members of the total coliform group live in the environment and wash into coastal waters on a daily basis. Therefore, the total coliform group does not accurately indicate sewage pollution. The presence of *E. coli*, although not a perfect indicator organism, is a better indicator of possible sewage contamination since the number of *E. coli* organisms in the mammalian gut is higher than in the environment in the continental United States. Since the Colilert-MW method

accurately determined the levels of *E. coli* within 24 h, we feel that it a powerful tool with which to rapidly assess possible fecal contamination in marine waters. This product could provide agencies that monitor coastal waters with a simple and rapid method for assessing the microbial conditions in nearshore beach and bathing waters.

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