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Detection of Coliform Bacteria and *Escherichia coli* by Multiplex Polymerase Chain Reaction: Comparison with Defined Substrate and Plating Methods for Water Quality Monitoring

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Multiplex polymerase chain reaction (PCR) and gene probe detection of target *lacZ* and *uidA* genes were used to detect total coliform bacteria and *Escherichia coli*, respectively, for determining water quality. In tests of environmental water samples, the *lacZ* PCR method gave results statistically equivalent to those of the plate count and defined substrate methods accepted by the U.S. Environmental Protection Agency for water quality monitoring and the *uidA* PCR method was more sensitive than 4-methylumbelliferyl-β-D-glucuronide-based defined substrate tests for specific detection of *E. coli*.

Ensuring the microbiological quality of potable waters to protect public health requires the detection of coliform bacteria and, in particular, *Escherichia coli* as indicators of human fecal contamination and possible associated human enteric pathogens (1). The U.S. Environmental Protection Agency recently revised its coliform-monitoring requirements to include the specific detection of *E. coli* (15, 16). Besides conventional viable plating and most-probable-number liquid culture methods for monitoring coliform bacteria, several defined substrate tests have been developed for both total-coliform and *E. coli* detection. These defined substrate tests include *Escherichia coli* broth (EC broth) containing 4-methylumbelliferyl-β-D-glucuronide (EC-MUG), nutrient agar with MUG, and lauryl tryptone broth with MUG (LTB-MUG) for the specific detection of *E. coli* following primary plating for the detection of presumptive coliforms (16, 17). The Colilert defined substrate-based test has been developed for the direct rapid detection from water samples of total coliform bacteria (based upon the demonstration of β-galactosidase enzyme activity by using the chromogenic substrate *ortho*-nitrophenyl-β-D-galactopyranoside [ONPG]) and of *E. coli* (based upon the demonstration of β-glucuronidase enzyme activity on the fluorogenic substrate MUG [MMO-MUG test]) (10–14, 18). Compared with conventional coliform detection methods—membrane filtration and the multiple tube fermentation test, methods which take 48 to 72 h to obtain confirmed results (1)—the MMO-MUG test takes only 24 h to determine the presence of coliform bacteria and *E. coli* in a water sample (12). Because some *E. coli* strains have a MUG^- phenotype (7, 9, 19) and sublethally injured *E. coli* may not be detected by MMO-MUG (8), MUG-based tests may underestimate the presence of *E. coli*.

We recently have reported on the development of polymerase chain reaction (PCR)-gene probe methods for the detection of total coliform bacteria and *E. coli* (2, 4, 6). Cleuziat and Robert-Baudouy (9) have also reported on PCR-gene probe detection of *E. coli*. The advantages of the PCR system include the sensitivity of PCR detection (which can detect single cells in 100-ml water samples), the specificity of PCR-gene probe detection for target microorganisms, the speed from the time of sample collection to the completion of analysis (which should take less than 6 h with nonradioactive probes), and the ability to simultaneously detect multiple target bacteria (which can include both general indicator species and a series of specific target pathogens). In the present study, we evaluated the effectiveness of multiplex PCR-gene probe detection of a portion of the *lacZ* gene of *E. coli* for total-coliform monitoring and a portion of the *uidA* gene for *E. coli* detection by comparing test results obtained from environmental samples by PCR with those determined by both defined substrate and conventional plating procedures.

To ensure the specificity of multiplex PCR for target organisms and equivalence with previous PCR coliform detection, which had shown that *lacZ* was diagnostic for total coliforms and that *uidA* was diagnostic for *E. coli* and *Shigella* spp., including MUG-negative *E. coli* (4, 6), multiplex PCR amplification with the *lacZ* and *uidA* genes as targets was performed on the 100 strains previously tested (4) and on an additional 29 *E. coli* environmental isolates—which included 7 EC-MUG-negative strains—and several isolates from environmental waters of other coliform bacteria, including *Klebsiella pneumoniae*, *K. oxytoca*, *K. ozaenae*, *Citrobacter freundii*, *Enterobacter cloacae*, *E. aerogenes*, and *E. agglomerans*. *Shigella sonnei* was detected. Several non-specific-target bacteria, including *Acinetobacter* sp. strains API Gr. 1 and Gr. 2, CDC VE1, a *Flavobacterium* sp., *Hafnia alvei*, a *Providencia* sp., *Serratia marcescens*, *S. liquefaciens*, a *Serratia* sp., and *Pseudomonas* spp., were also tested by multiplex PCR.

For multiplex PCR, a pair of 24-mer primers (5′-ATGAAA GCTGGCTACAGGAAGGCC-3′ and 5′-GGTTTATGCAGC AACGAGACGTCA-3′ [6]) located within the coding region of the *lacZ* gene of *E. coli* and a pair of 20- and 21-mer primers (5′-AAAACGGCAAGAAAAAGCAG-3′ and 5′-AC GCGTGGTTACAGTCTTGCG-3′) located within the *uidA* structural gene of *E. coli* (4) were used. Multiplex PCR was performed under conditions previously described (4). Both Southern and dot blot DNA-DNA hybridizations were carried out by following the procedures previously described (3, 5, 6), with one 25-mer probe for detection of *lacZ* (5′-CAG GATATGTGGCGGATGAGCGGCA) and another for *uidA* (5′-CCGAACACCTGGGTGGACGATATCA).

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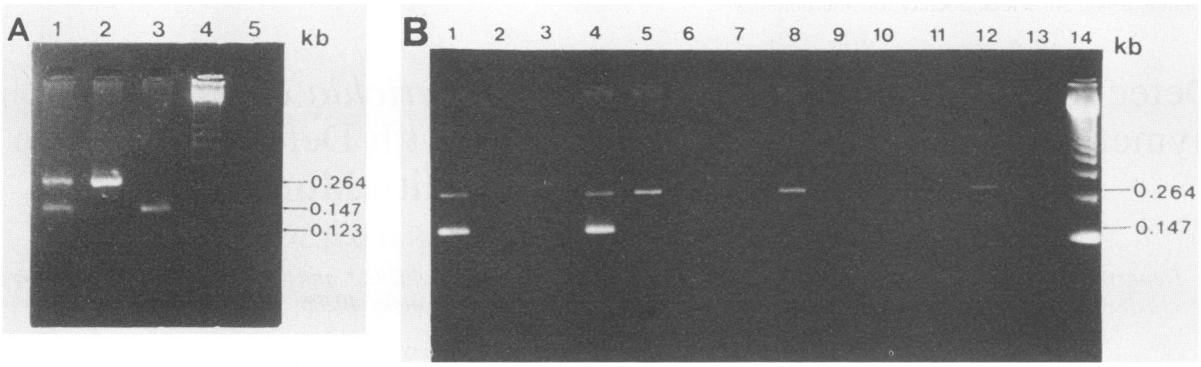

FIG. 1. Ethidium bromide-stained 4% NuSieve-SeaKem (1:3) agarose gel analysis of duplex PCR amplification of *E. coli* cells (A) and of the genomic DNAs of various bacterial strains isolated from environmental waters by using equimolar quantities (0.5 μM) of primers for the *lacZ* and *uidA* genes of *E. coli* as targets (B). (A) Lanes: 1, duplex PCR amplification using equimolar quantities (0.5 μM) of primers for the *lacZ* and *uidA* genes of *E. coli* as targets; 2, PCR amplification using 0.5 μM primers for the *lacZ* gene of *E. coli*; 3, PCR amplification using 0.5 μM primers for *uidA* gene of *E. coli*; 4, 123-bp DNA ladder as a size standard; 5, duplex PCR amplification using *lacZ* and *uidA* primers without any target DNA. (B) Lanes: 1, *Shigella sonnei*; 2, *H. alvei*; 3, *Serratia* sp.; 4, *E. coli*; 5, *K. oxytoca*; 6, *Acinetobacter* sp.; 7, *Pseudomonas fluorescens*; 8, *K. pneumoniae*; 9, *Providencia* sp.; 10, *S. liquefaciens*; 11, *Pseudomonas maltophilia*; 12, *Enterobacter aerogenes*; 13, *Chromobacterium* sp.; 14, 123-bp DNA ladder as a size standard.

In duplex amplification, equimolar quantities of primers yielded almost equally intense amplified DNA bands in an agarose gel for *lacZ* and *uidA* targets in *E. coli* (Fig. 1A). Interestingly, when *lacZ* and *uidA* targets were amplified separately, *lacZ* produced a more intense amplified DNA band than *uidA* (Fig. 1A). This result is possibly due to the fact that the *lacZ* target is larger than the *uidA* target or due to different GC contents of the two targets. A 0.147-kb DNA band in the agarose gel for the *uidA* target was detected for all *E. coli* and *Shigella* strains (Fig. 1B). No other coliform or noncoliform bacteria tested showed any amplification. A 0.264-kb DNA band was detected in the agarose gel for the *lacZ* gene target for all coliform bacteria; this band was not observed with any of the noncoliform bacteria (Fig. 1B). All 29 environmental *E. coli* isolates and the *Shigella sonnei* isolate showed positive amplified DNA bands for both *lacZ* and *uidA* targets when multiplex PCR amplification was performed. All other coliform bacteria amplified only the *lacZ* target. No amplification was detected with other bacteria. Amplification of *uidA* permitted detection of *E. coli* at the single-cell level (Fig. 2). Specific detection of *E. coli* and *Shigella* spp. makes the multiplex amplification method more useful for monitoring not only indicator bacteria but also the presence of waterborne pathogens in a single reaction. Importantly, MUG-negative *E. coli* strains, which include some enteropathogenic strains, were detected by PCR amplification of *uidA*, whereas these strains were not detected by MUG-based defined substrate tests.

To test the field applicability of multiplex PCR for coliform and *E. coli* monitoring in water, 90 water samples were analyzed by multiplex PCR, defined substrate, and conventional plate count methods. These samples included 9 water samples from the Louisville Water Co. drinking water purification systems (finished water), 36 dechlorinated finished water samples spiked with Ohio River water, 16 water samples from the effluent of a pilot-scale biologically active granular activated-carbon filter, 20 water samples from a treatment plant coagulation basin prior to disinfection, and 9 environmental isolates of coliform and noncoliform bacteria from the Ohio River.

For plate count determination of total coliforms, 100-ml water samples were filtered with 0.45-μm-pore-size filters (Millipore type HA) and the filters were placed on m-Endo broth (Difco) as described in *Standard Methods* (1). The presence of green-sheen-forming colonies (after 24 h of incubation at 35.5°C) capable of gas production in LTB and brilliant green bile lactose broth (Difco) within 48 h at 35.5°C was considered to indicate positive coliform CFUs. Defined substrate confirmation tests were performed by swabbing the surfaces of the membranes with sterile cotton and inoculating tubes of LTB containing 50 mg of MUG per liter (LTB-MUG) (Difco) and of EC broth containing 50 mg of MUG per liter (EC-MUG) (Difco). If the LTB-MUG and EC-MUG swab results were negative, the plate was considered *E. coli* negative (<1 CFU/100 ml). Total-coliform and *E. coli* MMO-MUG tests for each water sample also were performed in the presence-absence format by the Autoanalysis Colilert test (Access, Branford, Conn.). Randomly

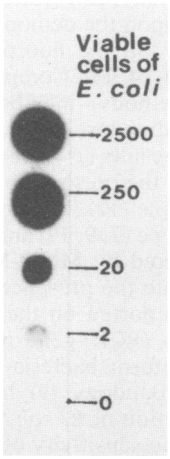

FIG. 2. Dot blot analysis after PCR amplification of serial dilutions of an 8-h *E. coli* culture grown in Luria-Bertani broth for 24 h at 35°C, using primers for *uidA* amplification. Viable cells were determined as CFUs on Luria-Bertani agar plates. A sample containing no *E. coli* cells was used as a negative control. Radiolabeled oligonucleotide probe for *uidA* was used for hybridization.

TABLE 1. Comparison of methods for detection of total coliform bacteria

Method and result	No. of results by *lacZ* PCR	
	+	−
Plate count		
+	56	4
−	9	21
Colilert ONPG		
+	61	3
−	4	22

TABLE 2. Statistical analysis of *lacZ* PCR versus the plate count and Colilert tests for total coliform bacterium detection

Test	Statistic[a] (*lacZ* PCR vs test)				
	Index of agreement	Cohen kappa	Pearson phi	McNemar	Binomial probability
m-Endo plate count	0.84	0.64	0.56	1.9	0.13
Colilert-ONPG	0.92	0.81	0.69	0.14	0.5

[a] None of the differences between methods are significant at $P < 0.05$.

selected colonies were isolated from the LTB-MUG, EC-MUG, and MMO-MUG tubes and plated onto MacConkey's agar for identification. Approximately 350 lactose-positive and -negative colonies were isolated and identified by using Analytab API 20E strips and conventional microbiological techniques.

For comparative testing of multiplex PCR, 100-ml water samples were filtered through 13-mm-diameter ethyl alcohol-presoaked Fluoropore membranes (Millipore type FHLP) by using Swinnex filter holders (Millipore). The filters containing the bacterial cells were transferred into GeneAmp reaction tubes with the cell-coated surface facing the inside of the tube. One hundred microliters of diethylpyrocarbonate-treated sterile distilled water was added to each tube, and the cells were released from the filter surface by pipetting the liquid repeatedly and by interrupted vortexing for several minutes. Without removing the filters, the bacterial cells in the tubes were lysed and the DNAs were released by repeated cycles (about 5 to 10 cycles) of freezing in an ethanol-dry ice bath and thawing at 40 to 50°C in a water bath (4).

Statistical analyses were performed as described for the evaluation of the Colilert test by Edberg et al. (11, 12). The statistical tests performed included the following: index of agreement, which measures the proportion of all samples for which there was agreement (proportion of both positive or both negative); Cohen kappa, which is a chance-corrected adjustment of the agreement; Pearson phi, which is an index of correlation; McNemar, which is a measure of whether one method produces a greater proportion of positive results when the results of the two methods differ; and the binomial probability, which measures the probability that when the results of two methods differ the difference is due to a 0.5 chance that either could be positive. The McNemar test is based upon the exact binomial distribution in cases in which the number of differences is less than or equal to 20 and the chi-square distribution when more than 20 differences occur; when the number of instances in which one test is positive and the other is negative is less than 5, caution must be used in interpreting the significance of the McNemar test if the calculated value is close to the critical value of significance, which is 3.8416. We considered a P value of <0.05 necessary to establish a statistically significant difference.

None of the finished water samples were coliform positive by any of the plate count, defined substrate, or PCR tests. Of the 90 water samples tested, there were 13 cases in which PCR and plate counts gave differing results and only 7 cases in which PCR and Colilert-ONPG tests differed for total-coliform detection (Table 1). The statistical analyses indicated that the plate count, defined substrate, and multiplex PCR methods were equivalent (Table 2). All cases in which the test results differed occurred when the plate counts showed <5 CFU/ml; at such low concentrations, sample-to-sample variability could account for the differences. The McNemar and exact binomial tests indicated that there was no significant difference in the distribution of positive and negative results when the tests differed, indicating that neither of the tests was more likely than the other to detect coliform bacteria; that is, the tests were statistically equivalent.

With regard to the specific detection of *E. coli*, there was somewhat better agreement between multiplex PCR and the LTB-MUG and EC-MUG confirmation tests, for which only 15 of 90 cases were not in agreement, than between the multiplex PCR and MMO-MUG tests, for which 21 of 90 cases differed (Table 3). The indices of agreement indicated approximately 80% correspondence when the PCR and MUG-based tests both detected *E. coli* or when both failed to detect *E. coli* (Table 4). As with the total coliform test comparisons, many of the differences occurred at low concentrations and may be due to differences among the samples analyzed.

The *uidA* PCR detection method, however, showed a significantly greater number of differences in which PCR was positive and the MUG test was negative; this was indicated by the significance of the McNemar test and the variance from the expected exact binomial distribution (Table 4). This result suggests that MUG-negative strains might be undetected by the defined substrate test and show a positive result by multiplex PCR. Considering all of the comparisons between the MUG-based defined substrate and *uidA* PCR tests for *E. coli*, if 15% of the *E. coli* in the samples were MUG negative, there would be a 99% probability, according to the exact binomial distribution, of obtaining the results found in this study. Independent tests on the occurrence of MUG-negative *E. coli* in the water samples used in this study

TABLE 3. Comparison of methods for detection of *E. coli*

Method and result	No. of results by *uidA* PCR	
	+	−
LTB-MUG		
+	39	4
−	11	36
EC-MUG		
+	38	3
−	12	37
MMO-MUG		
+	34	5
−	16	35

TABLE 4. Statistical analysis of *uidA* PCR versus defined substrate tests for *E. coli* detection

Method	Statistic[a] (*uidA* PCR vs test)				
	Index of agreement	Cohen kappa	Pearson phi	McNemar	Binomial probability
LTB-MUG	0.83	0.67	0.70	3.26*	0.06*
EC-MUG	0.83	0.67	0.68	5.4**	0.02**
MMO-MUG	0.77	0.54	0.58	5.8**	0.02**

[a] *, statistical difference nearly significant at $P < 0.05$; **, statistical difference significant at $P < 0.05$.

detected 15.8% confirmed MUG-negative *E. coli* in the Ohio River (19). Cleuziat and Robert-Baudouy (9) also reported the occurrence of 15% MUG-negative *E. coli* in their study and reported that the phenotypically β-glucuronidase-negative *E. coli* could be detected by a PCR-gene probe test based upon the *uid* gene. Testing of randomly selected bacteria isolated from the MUG confirmation and MMO-MUG tests showed that in 40% of the cases in which the multiplex PCR was positive for *E. coli* and the MUG tests were negative, *E. coli* was identified by the API test—with supplemental characterization to distinguish non-*E. coli Escherichia* spp.—although it had gone undetected despite its presence in the defined substrate tubes. Thus, the field evaluation appears to confirm the laboratory tests indicating that the *uidA* PCR method detects MUG-negative *E. coli* which are not detected by the MUG-based defined substrate methods.

In conclusion, multiplex PCR and gene probe detection of target *lacZ* and *uidA* genes appear to form the basis for the detection of total-coliform bacteria and *E. coli*, respectively. The *uidA* PCR method for *E. coli* detection appears to detect MUG-negative strains that may constitute 15% of the *E. coli* in the waters tested and that are not detected by the MUG-based defined substrate tests. A greater number of samples from various water sources will have to be analyzed to establish the efficacy of the PCR method for water quality monitoring before it can be proposed as an alternative to approved and pending plate count and defined substrate methods.

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REFERENCES

1. **American Public Health Association.** 1989. Standard methods for the examination of water and wastewater, 17th ed. American Public Health Association, Washington, D.C.
2. **Atlas, R. M., A. K. Bej, S. McCarty, J. DiCesare, and L. Haff.** Monitoring microbial pathogens and indicator microorganisms in water by using the polymerase chain reaction and gene probes. *In* J. R. Hall and G. D. Glysson (ed.), Monitoring water in the 1990's: meeting new challenges, ASTM STP 1102, in press. American Society for Testing and Materials, Philadelphia.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Sideman, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
4. **Bej, A. K., J. L. DiCesare, L. Haff, and R. M. Atlas.** 1991. Detection of *Escherichia coli* and *Shigella* spp. in water by using the polymerase chain reaction and gene probes for *uid*. Appl. Environ. Microbiol. **57**:1013–1017.
5. **Bej, A. K., M. H. Mahbubani, R. Miller, J. L. DiCesare, L. Haff, and R. M. Atlas.** 1990. Multiplex PCR amplification and immobilized capture probes for detection of bacterial pathogens and indicators in water. Mol. Cell. Probes **4**:353–365.
6. **Bej, A. K., R. J. Steffan, J. DiCesare, L. Haff, and R. M. Atlas.** 1990. Detection of coliform bacteria in water by polymerase chain reaction and gene probes. Appl. Environ. Microbiol. **56**:307–314.
7. **Chang, G. W., J. Brill, and R. Lun.** 1989. Proportion of β-glucuronidase-negative *Escherichia coli* in human fecal samples. Appl. Environ. Microbiol. **55**:335–339.
8. **Clark, D., D. B. Milner, R. Wolfe, M. S. Stuart, and B. Olson.** 1990. Comparative study of commercial MUG preparations with the standard methods membrane filtration fecal coliform test for the detection of *Escherichia coli* in water samples. *In* Proceedings of the American Water Works Association Water Quality Technology Conference. American Water Works Association, Denver.
9. **Cleuziat, P., and J. Robert-Baudouy.** 1990. Specific detection of *Escherichia coli* and *Shigella* species using fragments of genes coding for β-glucuronidase. FEMS Microbiol. Lett. **72**:315–322.
10. **Edberg, S. C., M. J. Allen, D. B. Smith, and M. J. Kris.** 1990. Enumeration of total coliforms and *Escherichia coli* from source water by the defined substrate technology. Appl. Environ. Microbiol. **56**:366–369.
11. **Edberg, S. C., M. J. Allen, D. B. Smith, and The National Collaborative Study.** 1988. 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. Environ. Microbiol. **54**:1595–1601.
12. **Edberg, S. C., M. J. Allen, D. B. Smith, and The National Collaborative Study.** 1989. National field evaluation of a defined substrate method for the simultaneous enumeration of total coliforms and *Escherichia coli* from drinking water: comparison with presence-absence techniques. Appl. Environ. Microbiol. **54**:1003–1008.
13. **Edberg, S. C., and M. M. Edberg.** 1988. A defined substrate technology for the enumeration of microbial indicators of environmental pollution. Yale J. Biol. Med. **61**:389–399.
14. **Edberg, S. C., and C. M. Kontnick.** 1986. Comparison of β-glucuronidase-based substrate systems for identification of *Escherichia coli*. J. Clin. Microbiol. **24**:368–371.
15. **Federal Register.** 1989. Drinking water: national primary drinking water regulations; total coliform proposed rule. Fed. Regist. **54**:27544–27567.
16. **Federal Register.** 1990. Drinking water: national primary drinking water regulations; analytical techniques coliform bacteria proposed rule. Fed. Regist. **55**:22752–22756.
17. **Katamay, M.** 1990. Assessing defined-substrate technology for meeting monitoring requirements of the total coliform rule. J. Am. Water Works Assoc. **82(9)**:83–87.
18. **Lewis, C. M., and J. L. Mak.** 1989. Comparison of membrane filtration and Autoanalysis Colilert presence-absence techniques for analysis of total coliforms and *Escherichia coli* in drinking water samples. Appl. Environ. Microbiol. **55**:3091–3094.
19. **McCarty, S., A. Bej, M. Perlin, and R. M. Atlas.** 1990. Defined substrate technology and polymerase chain reaction-gene probe for the detection of total coliform and *Escherichia coli* in water, p. 1519–1535. *In* Proceedings of the American Water Works Association Water Quality Technology Conference. American Water Works Association, Denver.