APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1997, p. 771–774 Vol. 63, No. 2
0099-2240/97/$04.00+0

Limitations of Highly Sensitive Enzymatic Presence-Absence Tests for Detection of Waterborne Coliforms and *Escherichia coli*

S. O. VAN POUCKE AND H. J. NELIS*

University of Ghent, Laboratory for Pharmaceutical Microbiology, B-9000 Ghent, Belgium

Received 17 July 1996/Accepted 11 November 1996

This study presents evidence for the unfeasibility of enzymatic presence-absence tests to detect one total coliform or one *Escherichia coli* organism in 100 ml of drinking water within a working day. The results of field trials with prototype chemiluminometric procedures indicated that the sensitivity-boosting measures that are essential to achieve the required speed compromise the specificity of the tests.

Current enzymatic presence-absence (P/A) tests for total coliforms (TC) and *Escherichia coli* in drinking water rely on the detection of β-galactosidase and β-glucuronidase, respectively (12). Commercial versions of this approach, including Colilert, Colisure, and Fluorocult, employ visual detection of a color and of fluorescence and take 18 to 24 h to complete. In view of the constant time pressure for drinking-water laboratories to report results (22), the question of whether the performance of enzymatic P/A procedures could be improved to the extent that they would be capable of detecting one TC or one *E. coli* organism in a water sample within a working day arises. Ideally, such testing should not take more than 3 h, as most laboratories receive their samples only in the late morning or the early afternoon. One obvious approach to enhance the sensitivity of enzymatic procedures would be to use instrumental rather than visual endpoint detection. For example, spectrophotometry reportedly reduced the duration of the original 24-h Colilert test by 2 to 6 h (17). Using fluorometry, Berg and Fiksdal (2) and Peterson et al. (13) were able to detect one fecal coliform per 100 ml of water within 7 h. However, the corresponding detection time for TC, which possess lower enzyme activity than *E. coli*, is substantially longer (2).

In a previous paper we described how the speed of an enzymatic test for TC in liquid medium could be considerably enhanced by conducting a constitutive β-galactosidase assay in the presence of a membrane permeabilizer after a brief propagation phase and by using instrumental, particularly chemiluminometric, endpoint detection (19). This prototype P/A test has now been applied in a field study.

Water samples. Natural water samples were collected from different sites in Flanders, Belgium. They included samples of surface water, partially purified water, distribution water, tap water, and well water. In some cases samples were diluted in order to obtain different levels of coliforms. A total of 80 samples (232 dilutions) and 32 samples (52 dilutions) were analyzed in the TC and the *E. coli* tests, respectively.

Seeded water samples were prepared by adding appropriate dilutions in peptone water of pure cultures grown overnight. *Klebsiella pneumoniae* 36 was kindly donated by D. van der Kooij (Kiwa, Nieuwegein, The Netherlands). All other strains were isolates from natural waters. Identification was done by the API 20E or the 20NE test (bio-Mérieux, Marcy l'Etoile, France). Overnight cultures were prepared in tryptic soy broth without dextrose (Difco Laboratories, Detroit, Mich.) but supplemented with 0.001% of isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma, St. Louis, Mo.).

Chemiluminometric P/A test for TC. Samples were filtered over 8.3-mm-diameter Durapore filters (0.45-μm pore size; Millipore, Bedford, Mass.) with a Biofiltration Unit (Celsis Lumac, Landgraaf, The Netherlands) connected to a vacuum pump. Filters were punched out of conventional 47-mm-diameter filters. Each filter was prewetted with approximately 10 ml of sterile distilled water before 100 ml of the sample was filtered. Simultaneously, at least three blank samples (i.e., sterile distilled water) were processed in the same way. Following filtration, the membrane filter was placed in a flat-bottom Lumacuvette (Celsis Lumac) containing 400 μl of Colicult (Celsis Lumac) supplemented with cefsulodin (Sigma; 10 μg/ml) and then vortex mixed. In cases in which a propagation phase preceded the actual enzyme assay, the cuvettes were preincubated at 35°C for 5 to 6 h.

To assay β-galactosidase activity, solutions (50 μl each) of polymyxin B sulfate (100 μg/ml in sterile distilled water; Sigma) and Galacton-Plus (Tropix, Bedford, Mass.) (90 μM in 0.05 M sodium phosphate buffer, pH 6.5, containing 10 mM magnesium chloride) were added to the cuvettes, which were subsequently incubated for another 45 min at 35°C.

Light emission was measured with a Lumac 2500 Biocounter (Celsis Lumac) after the addition of 100 μl of an alkaline enhancer (Emerald [Tropix], 1.6 mg/ml, diluted in 0.5 M aqueous piperidine) with the pump which is part of the luminometer. The responses (in relative light units) obtained for the samples (signal) and the blanks (background) were divided to yield signal-to-background (S/N) ratios, as described by Bronstein et al. (5). A sample was considered to yield a positive response when its corresponding S/N ratio was at least 2 (19).

Validation. Responses were interpreted based on U.S. Environmental Protection Agency guidelines (18). A loopful of the preincubation mixture was streaked on plate count agar (Difco) prior to the enzyme assay. After incubation for 24 h at 35°C, all colony types were subcultured until pure cultures were obtained. If the target organism could be isolated from the Lumacuvette, a response was considered to be true positive. A response was termed true negative when no target organisms were recovered. Target organisms were identified on the basis of Gram stain, oxidase, and *o*-nitrophenyl-β-D-galactopyranoside (ONPG; β-galactosidase assay) or *p*-nitrophenyl-β-D-glucuronide (β-glucuronidase assay) reactivity.

Reference bacteriological tests. TC were enumerated by the delayed-incubation TC procedure (12). Besides confirmation

* Corresponding author. Mailing address: University of Ghent, Laboratory for Pharmaceutical Microbiology, Harelbekestraat 72, B-9000 Ghent, Belgium. Phone: (32)-9-264 80 91. Fax: (32)-9-264 81 95. E-mail: Hans.Nelis@rug.ac.be.

TABLE 1. Agreement between the chemiluminometric P/A test for TC and the reference procedure

Test	No. of responses		
	Negative	Positive	Total
Reference procedure[a]	128	104	232
P/A test	75	157	232

[a] Delayed-incubation TC procedure.

in lauryl tryptose broth (48 h, 35°C; Difco) and brilliant green bile lactose broth (24 h, 35°C; Difco), typical colonies were subcultured on tryptic soy agar (TSA; Difco) and verified for oxidase and ONPG reactivity.

Aeromonas spp. were enumerated on trehalose ampicillin agar (16) at 35°C. Typical colonies appeared as yellow on a dark blue background after incubation for 24 h.

The total count of β-galactosidase-containing bacteria was determined at 22 and 35°C after membrane filtration and incubation on R2A agar (Oxoid, Ltd., Basingstoke, United Kingdom) (72 h) and TSA (48 h), respectively, each containing 0.1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Biosynth AG, Staadt, Switzerland) per ml.

Gram-negative rods were identified on the basis of oxidase reaction and the API 20E or the 20NE test. Gram-positive cocci were characterized to genus level with the catalase test, whereas gram-positive rods were not further differentiated. All isolates were verified for ONPG reactivity.

Optimized chemiluminometric P/A test for TC in seeded water samples. With the original chemiluminometric P/A test, approximately 7 to 10 h was required to detect the presence of 1 CFU of TC seeded in water (19). Enhanced sensitivity has subsequently been achieved with a new, purposely developed growth and assay medium (Colicult) in conjunction with a superior chemiluminogenic substrate (Galacton-Plus) (20) and with scaled-down sample concentrations, as first described in this paper. Colicult was further supplemented with cefsulodin to suppress the growth of *Aeromonas* spp., as recommended by Brenner et al. (4) and Alonso et al. (1). The optimized procedure is capable of detecting one TC seeded in 100 ml of water in a total time (i.e., sample concentration time plus preincubation time plus enzyme assay time) of 6 h (*E. coli*, *K. pneumoniae*, and *Enterobacter intermedius*) to 7 h (*Citrobacter freundii*). Although the long-term goal of a 3-h test could not be reached with spiked waters, a field trial to test the performance of the prototype method with naturally contaminated water samples appeared to be warranted.

Specificity. As shown in Table 1, no agreement was obtained between the chemiluminometric and the reference procedures, the difference being significant at a P value of 0.001 (nonparametric chi-square test for proportional data [21]). This disagreement was mainly caused by an extremely high false-positive rate of 56.7%, resulting in a specificity of only 40.3%. False-positive responses were the result of the presence of considerable numbers of β-galactosidase-containing noncoliforms (Table 2). On TSA supplemented with X-Gal and incubated at 35°C, the β-galactosidase-containing strains most frequently isolated from water included *Aeromonas* spp. (37%), *Staphylococcus* spp. (22%), *Bacillus* spp. (22%), and *Pseudomonas* spp. (15%). β-Galactosidase-positive species recovered at 22°C on R2A agar containing X-Gal mostly belonged to the genera *Flavobacterium* (19%), *Pseudomonas* (15%), and *Xanthomonas* (11%). Although none of these species were found to propagate at 35°C in Colicult supplemented with cefsulodin, their β-galactosidase activity was mostly preserved during preincubation (Table 3). However, the extent of the interference of nontarget bacteria would be underestimated on the basis of the X-Gal-positive plate counts at 35°C. The β-galactosidase-containing species additionally recovered at 22°C (plate counts differed by 2 to 3 log units from those recovered at 35°C, as shown in Table 2) will indeed also react "passively" in the sensitive constitutive enzyme assay. The above three phenomena, i.e., the absence of propagation of nontarget bacteria in Colicult supplemented with cefsulodin, the maintenance of

TABLE 2. Recovery of X-Gal-positive bacteria and *Aeromonas* organisms and S/N ratio in the chemiluminometric P/A test for TC before and after preincubation of samples containing no TC[a]

Sample	Type	No. of X-Gal-positive bacteria (CFU/100 ml) recovered at:		No. of *Aeromonas* organisms (CFU/100 ml)	S/N ratio at:	
		35°C	22°C		0 h	6 h
1	DW	26	2,100	19	1.2	1.3
2	DW	6	TNTC	0	1.4	1.0
3	DW	414	510	10	1.7	2.2
4	DW	1	342	2	2.9	ND
5	DW	0	118	0	3.0	ND
6	TW	0	0	0	3.1	ND
7	TW	0	1,000	0	3.4	2.8
8	TW	0	6	0	5.0	ND
9	TW	730	412	0	5.1	6.6
10	DW	66	5,000	9	5.5	ND
11	TW	1	1,800	0	6.0	6.0
12	DW	0	2,200	0	8.0	ND
13	TW	2	318	0	8.0	3.9
14	TW	3	36,000	0	9.0	9.0
15	DW	8	500	0	12.6	10.0
16	DW	6	424	7	13.0	15.0
17	DW	342	TNTC	165	24.7	ND
18	DW	0	20,000	0	25.8	20.1
19	DW	42	TNTC	35	27.0	35.0
20	TW	12	TNTC	8	29.0	ND
21	TW	0	11	1	30.0	ND
22	DW	60	4,240	111	42.0	45.0
23	DW	48	TNTC	15	50.0	ND
24	TW	ND	TNTC	100	74.0	ND
25	TW	ND	TNTC	23	69.0	ND
26	DW	396	20,000	236	113.0	42.0
27	DW	146	20,000	22	400.0	300.0

[a] DW, distribution water; TW, tap water; ND, not determined; TNTC, too numerous to count.

TABLE 3. Cell number and chemiluminescent response (S/N ratio) of β-galactosidase-containing nontarget organisms isolated from natural water samples, before and after incubation in Colicult supplemented with cefsulodin (10 μg/ml)

Organism	No. of cells (CFU/ml)[a] at:		Response (S/N ratio) at:	
	0 h	6 h	0 h	6 h
Staphylococcus spp.	4×10^4	6×10^4	1.5	2.7
Bacillus spp.	1×10^3	3×10^0	35.1	3.0
Flavobacterium indologenes	4×10^6	3×10^4	2.0	1.9
Sphingomonas paucimobilis	4×10^3	5×10^1	35.4	43.4
Aeromonas hydrophila	2×10^4	2×10^2	13.6	12.8
Aeromonas caviae	1×10^4	2×10^1	2.0	3.7
Aeromonas sobria	4×10^4	4×10^2	3.2	18.9
Vibrio parahaemolyticus	2×10^4	1×10^2	3.9	2.9

[a] As determined on TSA at 22°C.

TABLE 4. Recovery of X-Gluc-positive nontarget bacteria from natural water samples on TSA at 35°C and R2A agar at 22°C in relation to the corresponding count for *E. coli*

E. coli count (CFU/100 ml)	No. of X-Gluc-positive nontarget bacteria (CFU/100 ml) recovered at:	
	35°C	22°C
5	75	13,200
10	14	93
2	1	38
0	180	160
0	20	30,000
0	22	3,600
40	41	62
4	4	170,000
0	50	20,000
0	50	101,000

their β-galactosidase activity in the course of preincubation, and the "passive" nature of the interference, were confirmed with natural water samples containing no TC (Table 2). High-level responses had already been obtained at time zero, and these remained roughly unchanged after preincubation. In the few samples yielding a negative or a very low X-Gal-positive plate count, high responses may have been caused by the presence of viable but nonculturable bacteria. "Passive" interference of high numbers of nontarget bacteria, i.e., exceeding 20,000 CFU/ml, has also been reported for defined-substrate tests (7–10). However, the latter include an extended growth phase of 18 to 24 h, which leads to the predominance of initially low numbers of actively growing TC over the nongrowing interferences. In contrast, the growth phase of the present test is too short to shift the equilibrium towards the target bacterium if the latter occurs in low numbers. For example, if sample 14 (Table 2), containing 3.6×10^4 X-Gal-positive bacteria, harbored one TC and six generations occurred in 6 h, the final number of target bacteria would only be 64, affording a ratio of 563:1 in favor of the interfering species.

The rationalization of the high false-positive error in the TC test was supported by similar findings with a newly developed chemiluminometric procedure for *E. coli*. The latter differs from the TC test only in that it uses β-glucuronidase as a marker enzyme and Glucuron (Tropix) (90 μM in 0.05 M sodium phosphate buffer, pH 6.5, containing 10 mM magnesium chloride) as a chemiluminogenic substrate instead of Galacton-Plus. Eighteen samples containing 1 to 10 CFU of *E. coli* per 100 ml (mean ± standard deviation = 3.3 ± 2.7), as determined on m FC agar (Difco) and C-EC agar (Biolife, Milan, Italy) at 44.5°C, already yielded S/N ratios of 53.5 ± 97.7 (mean ± standard deviation) at time zero. These high-level responses could not have been solely caused by such low numbers of the target bacterium, because the chemiluminometric test for *E. coli* has a limit of detectability (S/N ratio of 2) of 2 CFU/100 ml after 9 h of preincubation. Hence, the presence of other β-glucuronidase-containing nontarget bacteria that interfere "passively" was suspected and experimentally confirmed on nonselective media containing 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc, 0.1 mg/ml; Biosynth AG) as a chromogenic substrate, i.e., TSA (incubated at 35°C) and R2A agar (22°C). As for TC, the counts on R2A agar at 22°C were 2 to 3 log units higher than those on TSA at 35°C (Table 4). The interference of β-glucuronidase-containing nontarget bacteria was further confirmed with water samples ($n = 14$) that contained no *E. coli* but did yield positive responses, both at time zero (S/N ratio = 3.7 ± 2.9) and after 5 h of preincubation (S/N ratio = 3.3 ± 2.9). The presence in water of β-glucuronidase-containing *Shigella* spp. (3), *Flavobacterium* spp. (14), *K. pneumoniae*, *C. freundii*, and *Aeromonas hydrophila* (6) has been reported. In this study, most β-glucuronidase-containing isolates belonged to the genus *Bacillus.*

Sensitivity. The chemiluminometric test for TC suffered not only from low specificity but also from poor sensitivity (81.9%). Reported sensitivities of defined substrate P/A tests range from 35% (15) to 94% (10). An important difference between the approach in this study and the defined substrate technology (11) lies in the extremely short incubation time of the former. A propagation phase of only 6 h obviously must fail to recover many stressed or injured target organisms from water.

Overall, false-negative responses were found mostly with samples containing fewer than 10 TC/100 ml. However, given the above-mentioned nonspecificity of the test, the sensitivity figures may even have been overestimated, because no distinction could be made between the responses from target and nontarget bacteria. Furthermore, the addition of cefsulodin as an extra inhibitor to improve the specificity increased the undetected-target error rate from 7.2 to 18.1%, possibly because of the higher susceptibility of injured TC to this antibiotic.

Conclusion. At first, the negative outcome of a field study of a new prototype enzymatic P/A test for TC may not appear to be a finding of great interest and may not even seem worth reporting. However, the consequences of our observations for the feasibility of "very rapid" enzymatic P/A tests in general do appear to be far-reaching. Based on the interpretation of the high false-negative and false-positive errors of the prototype TC method, we claim that the sensitivity and particularly the specificity problems encountered are probably inherent in any enzymatic P/A test that has the potential of detecting one target organism in less than 8 h. The paradox is that the extreme sensitivity-boosting measures, particularly chemiluminometric endpoint detection and sample concentration, are absolutely essential to the accomplishment of the objective, but they enhance the detectability of β-galactosidase- or β-glucuronidase-containing nontarget bacteria so much that they start to interfere in the constitutive enzyme assay. Thus, a fundamental conflict arises between the requirements of assay sensitivity (and speed) on the one hand and target specificity on the other. A preincubation period of 5 to 6 h is too short to create a predominance of low numbers of TC or *E. coli*, either injured or not, over an excess of other bacteria that also contain the marker enzyme. This highly unfavorable ratio between target and nontarget bacteria would be even more pronounced in a 3-h P/A test. Such a procedure would hardly involve bacterial propagation and, consequently, would lose all specificity for the target organism. Furthermore, it would no longer have the potential of detecting 1 CFU of TC or *E. coli* per 100 ml. It is concluded that, unless specificity can be improved, e.g., by isolation of the target bacteria, "very rapid" enzymatic P/A tests will not be applicable in the field because the detection sensitivity and target specificity requirements cannot be reconciled.

This work was part of research project 5-9 of the Flemish Centre for Water Research (SVW, Antwerp) and was jointly supported by SVW and Celsis Lumac.

We thank Saskia Dhondt and Els De Keijser for excellent technical assistance. We are indebted to the Vlaamse Maatschappij voor Watervoorziening (VMW), the Antwerpse Waterwerken (AWW), the Tussengemeentelijke Maatschappij der Vlaanderen voor Watervoorziening (TMVW), and the Provinciale en Intercommunale Drinkwatermaatschappij der Provincie Antwerpen (PIDPA) for participation in

the field trial. The help of J. Paulussen (VMW) in the calculation of the statistics is greatly appreciated.

REFERENCES

1. **Alonso, J. L., I. Amoros, and M. A. Alonso.** 1996. Differential susceptibility of aeromonads and coliforms to cefsulodin. Appl. Environ. Microbiol. **62:** 1885–1888.
2. **Berg, J. D., and L. Fiksdal.** 1988. Rapid detection of total and fecal coliforms in water by enzymatic hydrolysis of 4-methylumbelliferone-β-D-galactoside. Appl. Environ. Microbiol. **54:**2118–2122.
3. **Berger, S. A.** 1994. Increased protection afforded by the defined substrate technology Colilert system by its ability to detect *Shigella* β-glucuronidase. Lett. Appl. Microbiol. **19:**53–56.
4. **Brenner, K. P., C. C. Rankin, Y. R. Roybal, G. N. Stelma, Jr., P. V. Scarpino, and A. P. Dufour.** 1993. New medium for the simultaneous detection of total coliforms and *Escherichia coli* in water. Appl. Environ. Microbiol. **59:**3534–3544.
5. **Bronstein, I., B. Edwards, and J. C. Voyta.** 1989. 1,2-Dioxetanes: novel chemiluminescent enzyme substrates. Application to immunoassays. J. Biolumin. Chemilumin. **4:**99–111.
6. **Ciebin, B. W., M. H. Brodsky, R. Eddington, G. Horsnell, A. Choney, G. Palmateer, A. Ley, R. Joshi, and G. Shears.** 1995. Comparative evaluation of modified m-FC and m-TEC media for membrane filter enumeration of *Escherichia coli* in water. Appl. Environ. Microbiol. **61:**3940–3942.
7. **Covert, T. C., L. C. Shadix, E. W. Rice, J. R. Haines, and R. W. Freyberg.** 1989. Evaluation of the Autoanalysis Colilert test for detection and enumeration of total coliforms. Appl. Environ. Microbiol. **55:**2443–2447.
8. **Cowburn, J. K., T. Goodall, E. J. Fricker, K. S. Walter, and C. R. Fricker.** 1994. A preliminary study of the use of Colilert for water quality monitoring. Lett. Appl. Microbiol. **19:**50–52.
9. **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.
10. **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 detection of total coliforms and *Escherichia coli* from drinking water: comparison with presence-absence techniques. Appl. Environ. Microbiol. **55:**1003–1008.
11. **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.
12. **Greenberg, A. E., L. S. Clesceri, and A. D. Eaton (ed.).** 1992. Standard methods for the examination of water and wastewater, 18th ed. American Public Health Association, Washington, D.C.
13. **Peterson, S. M., S. C. Apte, C. M. Davies, I. Johns, P. Fitch, G. Swan, T. Prus-Wisniowski, and G. Skyring.** 1995. Rapid automated monitoring of coliforms in reticulated water. *In* Proceedings of the Australian Water and Wastewater Association 16th Federal Convention. Australian Water and Wastewater Association, Sydney.
14. **Petzel, J. P., and P. A. Hartman.** 1986. A note on starch hydrolysis and β-glucuronidase activity among flavobacteria. J. Appl. Bacteriol. **61:**421–426.
15. **Schets, F. M., G. J. Medema, and A. H. Havelaar.** 1993. Comparison of Colilert with Dutch standard enumeration methods for *Escherichia coli* and total coliforms in water. Lett. Appl. Microbiol. **17:**17–19.
16. **Soontjens, C.** 1994. Thesis. Hoger Instituut voor Technische en Paramedische Wetenschappen, Geel, Belgium.
17. **Standridge, J. H., S. M. Kluender, and M. Bernhardt.** 1992. Spectrophotometric enhancement of MMO-MUG (Colilert) endpoint determination, p. 157–162, p. 183–189. *In* Proceedings of the Water Quality Technology Conference. American Water Works Association, Toronto, Canada.
18. **U.S. Environmental Protection Agency.** 1993. Protocol for nationwide approval of new or revised methods for total coliforms, fecal coliforms, and/or *E. coli*, in national drinking water monitoring. Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio.
19. **Van Poucke, S. O., and H. J. Nelis.** 1995. Development of a sensitive chemiluminometric assay for the detection of β-galactosidase in permeabilized coliform bacteria and comparison with fluorometry and colorimetry. Appl. Environ. Microbiol. **61:**4505–4509.
20. **Van Poucke, S. O., and H. J. Nelis.** Effects of the composition of bacteriological growth media on a chemiluminometric assay of β-galactosidase in *Escherichia coli*. J. Biolumin. Chemilumin., in press.
21. **Wardlaw, A. C.** 1985. Practical statistics for experimental biologists, p. 85–86 and 100–102. John Wiley & Sons, Chichester, England.
22. **Young, P.** 1996. Safe drinking water: a call for global action. ASM News **62:**349–352.