APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1996, p. 3881–3884 Vol. 62, No. 10
0099-2240/96/$04.00+0

Evaluation of Enterolert for Enumeration of Enterococci in Recreational Waters

GARY E. BUDNICK,* ROBERT T. HOWARD, AND DONALD R. MAYO

Division of Laboratory Services, State of Connecticut Department of Public Health, Hartford, Connecticut 06106

Received 27 March 1996/Accepted 23 July 1996

Enterolert (IDEXX Laboratories Inc., Westbrook, Maine), a semiautomated, most probable number method for enumeration of enterococci, was compared with the standard membrane filter method by parallel testing of 138 marine and freshwater recreational bathing water samples. No statistically significant difference and a strong linear correlation were found between methods. Culturing of 501 Enterolert test wells resulted in false-positive and false-negative rates of 5.1 and 0.4%, respectively. Less time for setup, incubation (24 versus 48 h), and reading of Enterolert permits more efficient monitoring of recreational bathing areas.

Many tidal marine and inland freshwater areas are used for recreational bathing purposes. Under certain conditions, these areas may be adversely affected by fecal pollution from a variety of sources including municipal wastewater and raw sewage effluent from treatment plants, private septic disposal systems, and stormwater runoff. In addition to being aesthetically unacceptable, there is a measurable and significant risk of acute gastroenteritis associated with exposure to water contaminated with fecal waste (5). The sanitary quality of recreational bathing areas is therefore routinely monitored by public health authorities for the presence of microbiological contamination, and studies have indicated that enterococci are the best recreational water indicators for this purpose (4, 7).

The mE method of Levin et al. (13) is the standard membrane filter (MF) method used to test recreational bathing waters for enterococcal levels (1, 17), and although this method was shown to efficiently recover enterococci from marine and estuarian waters, the false-positive and false-negative rates were found to be 10.0 and 11.7%, respectively. A semiautomated most probable number (MPN) method, Enterolert, has been developed, one that can enumerate enterococci in bathing waters in significantly less time than the MF procedure while requiring less manipulation and quality control testing. The test utilizes a nutrient indicator substrate, 4-methylumbelliferone-β-D-glucoside, that fluoresces when metabolized by enterococci. Methylumbelliferyl derivatives have the advantage of being highly sensitive and specific, noncarcinogenic, and easily detected with UV light sources. Consequently, test systems using methylumbelliferyl substrates have been designed for environmental monitoring of fresh and marine waters for fecal pollution (2, 11). Enterolert is based on Defined Substrate Technology (9), which has been used successfully to test fresh (6, 8) and marine (16) waters for fecal indicator organisms. In this study, we have evaluated the Enterolert methodology and compared it with the standard MF technique with samples obtained from routinely monitored tidal marine and freshwater recreational bathing areas. The 138 fresh and marine water samples used in this study were collected by the Connecticut State Department of Environmental Protection/Bureau of Water Management and Monitoring and local health departments for routine monitoring of tidal marine and inland freshwater public bathing areas. Dates of collection were from 12 June 1995 through 11 September 1995, and all samples were kept on ice during transport to the laboratory and tested within 30 h of collection.

Test procedures. Enterolert provides an MPN result based on the presence or absence of fluorescence in 51 individual wells each containing a sample-nutrient indicator mixture. A 1:10 dilution of the test water sample was prepared (90 ml of sterile deionized water plus 10 ml of sample) in a sterile polystyrene vessel. One package of powdered Enterolert reagent was then added to the vessel, and the sample-reagent combination was mixed and then poured into a Quanti-Tray, a sterile plastic disposable panel containing 51 wells. The tray was then mechanically sealed, distributing the mixture into the wells, and incubated for 24 h at 41.0 ± 0.5°C. Test results were read in a dark environment by placing the Quanti-Tray under and within 5 in. (ca. 12 cm) of a 365-nm-wavelength UV light with a 6-W bulb (Spectroline Model EA-160; Spectronics Corporation, Westbury, N.Y.), and the number of positive wells was counted. Any fluorescence in a well was considered a positive reaction for that well and indicated the presence of enterococci. On the basis of the number of positive wells, MPN tables and a dilution factor were used to determine the enterococcal density per 100 ml of sample. Quanti-Tray wells showing no fluorescence were considered negative for enterococci. The MF procedure was performed as described in *Standard Methods for the Examination of Water and Wastewater* (1) and is recognized by the U.S. Environmental Protection Agency as acceptable for testing recreational bathing waters (3, 17). The procedure provides a direct count of bacteria based on the development of colonies on the surface of an MF. Ten milliliters of the thoroughly mixed, undiluted, test water sample was filtered through a 0.45-μm-pore-size, gridded, sterile hydrophobic MF (Millipore Corporation, Bedford, Mass.), and the filter was transferred to mE agar medium (Difco Laboratories, Detroit, Mich.) and incubated for 48 h at 41.0 ± 0.5°C. Filters with any pink to red presumptive enterococcal colonies on the surface were confirmed with esculin-iron agar (Difco Laboratories). In this method, enterococcal colonies develop a black or reddish brown precipitate on the underside of the filter. Positive enterococcal colonies were counted, and the enterococcal density per 100 ml of sample was calculated with the dilution factor. For each individual sample, MF and Enterolert testing was performed with portions from the same mixed-sample bottle.

Statistical analysis. A comparison of the Enterolert and MF

* Corresponding author. Mailing address: Division of Laboratory Services, State of Connecticut Department of Public Health, 10 Clinton St., Hartford, CT 06106. Phone: (860) 566-2717. Fax: (860) 566-7813.

methods for statistical correlation was done by InStat, a statistical software program (GraphPad Software, Inc.). Analysis was done on results of the total sample population in addition to the separate freshwater and marine categories.

(i) Paired *t* test. Analysis of the combined sample population showed no statistically significant difference between the Enterolert and MF methods ($P = 0.63$). Results classified by sample type showed a greater difference between the two methods when freshwater versus marine water samples were evaluated.

(ii) Linear correlation. A strong positive correlation between both methods for the total sample group ($r = 0.97$) was found and also when the freshwater and marine samples were analyzed separately ($r = 0.99$ for marine samples; $r = 0.76$ for freshwater samples).

Culturing of Quanti-Tray wells and enterococcal confirmation. Optimum growth of enterococci occurs at 35°C, and most strains of enterococci grow at 45°C, tolerate 6.5% NaCl, and hydrolyze esculin in the presence of 40% bile salts (bile-esculin [BE] medium) (10, 15). On the basis of these characteristics, a protocol was designed to test positive and negative Quanti-Tray wells for the presence of enterococci. A mechanical pipette (Medical Laboratory Automation, Inc., Pleasantville, N.Y.) with a sterile, disposable polypropylene tip was used to pierce each Quanti-Tray well and aspirate approximately 50 μl of test broth. This inoculum was streaked to a BE agar plate and incubated for 24 to 48 h at 35°C. No growth after 48 h was considered a negative test for enterococci. The presence of small colonies surrounded by a black halo was considered presumptive identification for enterococci. Enterococci were isolated from mixed flora by restreaking the primary growth to BE agar either directly from the BE plate or from a suspension of growth in Trypticase soy broth. If after four passages, presumptive enterococci were not found, the well was considered negative. Enterococcal colonies were then confirmed by inoculating presumptive growth from the BE plate into brain heart infusion broth containing 6.5% NaCl (BHI–6.5% NaCl) and incubating for 24 to 48 h at 45°C. Turbidity confirmed the presence of enterococci. Because growth of some strains of enterococci may be slow or even absent in the presence of 6.5% NaCl and some strains do not grow at all at 45°C (10, 15), presumptive enterococcal isolates not growing at 45°C were inoculated onto 5% sheep blood agar plates and incubated for 24 to 48 h at 35°C. Growth from the sheep blood agar plates was then reinoculated into BHI–6.5% NaCl and incubated at 35°C. Turbidity at this temperature confirmed the presence of enterococci. This protocol was used to culture a total of 501 Quanti-Tray wells (256 positive and 245 negative) for enterococci, and the results were used to determine the sensitivity and specificity of Enterolert.

Positive wells. Broth from each of 256 positive wells was inoculated onto BE agar plates and incubated at 35°C. Growth was observed from all but three wells, and these were considered false-positive reactions. Initial screening results (BE agar reactions and BHI–6.5% NaCl growth studies) clearly demonstrated that enterococci were present in 211 of the 253 remaining positive wells. Inconclusive preliminary test results on the final 42 positive well isolates required that identification testing be done to confirm them as enterococci. Thirty-two of these strains were BE positive and had colony morphology consistent with most enterococci and yet did not grow in BHI–6.5% NaCl at 45°C. On the basis of automated and standard biochemical testing, all 32 of these strains were identified as enterococcal species: 18 as *Enterococcus casseliflavus* or *E. gallinarum*, 11 as *Enterococcus faecium*, 1 as *E. casseliflavus*, 1 as *E. gallinarum*, and 1 as *Enterococcus durans*. In all, enterococci were isolated from 243 positive wells. The final 10 positive well isolates were not enterococci, on the basis of inconsistent morphology on BE agar and Gram stain reactions, and were then further identified to genus and species. Also, in order to determine if these nonenterococcal strains were in fact the cause of the original fluorescence in their respective positive wells, all 10 isolates were also reinoculated to Enterolert and BE agar. Five of the 10 nonenterococcal isolates were positive for both tests (suggesting that they were the cause of the original fluorescence in the positive Quanti-Tray wells) and were identified as *Proteus vulgaris* (two isolates), *Serratia marcescens*, *Sphingomonas paucimobilis* [*Pseudomonas paucimobilis*], and a *Flavobacterium* sp. The remaining five nonenterococcal isolates were negative for Enterolert upon reinoculation, thereby confirming that they were not the cause of the original Quanti-Tray well fluorescence. These isolates were identified as *Providencia stuartii*, *Roseomonas fauriae*, *Bacillus sphaericus*, *Vibrio parahaemolyticus*, and *Pasteurella multocida*. Since enterococci were not isolated from any of these 10 positive wells, all were considered true false-positive wells, whether or not the Enterolert test was positive following reinoculation. It was therefore determined that 243 of the 256 positive Quanti-Tray wells contained enterococci.

Negative wells. Of the 245 negative wells inoculated onto BE agar plates, 244 either did not grow or were BE negative. One well contained *Enterococcus faecalis*. The results of the positive and negative well confirmation testing are summarized as follows: of the 256 positive wells, 243 (95.3%) were true positive and 13 (5.1%) were false positive, and of the 245 negative wells, 244 (99.6%) were true negative and 1 (0.4%) was false negative. On the basis of these results, Enterolert has a sensitivity of 99.6% and a specificity of 94.9%.

Microbiological quality assessment of sample group. To evaluate the effect that using different test procedures would have on microbiological acceptability, samples were first separated into marine and freshwater groups. Then, on the basis of the MF and MPN results, and with established maximum density criteria, samples were categorized as to acceptability for recreational bathing use. The Environmental Protection Agency has issued recommended criteria for a running geometric mean (RGM; ≥5 sample results in a 30-day period) and single-sample (SS) maximum allowable densities of enterococci in fresh and marine water used for recreational bathing purposes (18). The maximum RGM values (freshwater, 33/100 ml; marine, 35/100 ml) were developed by using currently accepted illness rates and calculated by using equations developed from studies of freshwater and marine bathing beaches (4, 7). By using these calculated maximum RGM values, a range of SS density recommendations was then determined on the basis of degree and frequency of use of recreational areas, with more intensively used areas being assigned lower cutoff values. The most restrictive recommended maximum SS densities (61/100 ml for freshwater and 104/100 ml for marine samples) are for designated beach areas, i.e., areas that are frequently lifeguard protected, provide parking and other public access, and are heavily used by the public. Using these Environmental Protection Agency recommendations as a guideline, the State of Connecticut monitored and compared results from select marine and freshwater bathing areas throughout one season and found that acceptable closure rates could be maintained by using the more protective freshwater limits for marine bathing areas. Therefore, the State of Connecticut has established a restrictive maximum RGM of 33/100 ml and a SS density of 61/100 ml as cutoff limits for both marine and freshwater bathing areas. Although the SS density is more affected by seasonal and incidental variation, for the

TABLE 1. Comparison of recreational bathing water samples based on sample type and test method result

Test	Water quality					
	No. of samples not acceptable[a] (%)			No. of samples acceptable (%)		
	Fresh (n = 43)	Marine (n = 95)	Total (n = 138)	Fresh (n = 43)	Marine (n = 95)	Total (n = 138)
MF	7 (16.3)	12 (12.6)	19 (13.8)	36 (83.7)	83 (87.4)	119 (86.2)
Enterolert	5 (11.6)	10 (10.5)	15 (10.9)	38 (88.4)	85 (89.5)	123 (89.1)

[a] For primary contact recreational use, >61 enterococci per 100 ml; SS standard for bathing water quality.

purposes of comparing the bathing water samples tested in this study, we have used only the SS cutoff value of 61 enterococci per 100 ml to classify recreational bathing water samples as either unacceptable (exceeds the SS standard) or acceptable for recreational use (Table 1).

Testing by the Enterolert method resulted in a greater percentage of samples with acceptable test results when the combined population was considered (89.1 versus 86.2% for the MF method). Nineteen of the 138 samples were judged to be unacceptable if the MF was used as the test of choice whereas only 15 samples would be unacceptable if the Enterolert procedure was used. When looking at the marine and freshwater categories separately, using Enterolert resulted in a higher percentage of acceptable bathing water samples in each. For marine samples, the microbiological quality was judged acceptable 2.1 and 4.7% more frequently for marine and freshwater samples, respectively. For all 138 samples taken as a whole, the mean enterococcal density per 100 ml of sample was 40.0 and 34.0 for the MF and Enterolert procedures, respectively. For the marine sample group, the mean test result was comparable for both methods: 43.8 for MF and 42.3 for Enterolert. The freshwater sample result means were more variable, 31.9 for MF and 15.6 for Enterolert.

Effect of test methodology on sample quality determination. Given the MF and MPN test results for each individual sample and using the established maximum SS enterococcal density, we were specifically interested in samples with conflicting results and subsequent differences in microbiological acceptability. Numerical enterococcal counts were first obtained for each sample by parallel testing. Then by referring to standard tables showing the upper and lower 95% confidence limits corresponding to each count for the MF and 51-well Quanti-Tray MPN methods, a range of values for each test result was established. If the 95% confidence limit ranges of the two test method results corresponded in any way, then the test results were considered to be in agreement. Of the 138 bathing water samples tested, 13 (9.4%) had test results that either disagreed (no correlation between the 95% confidence limit ranges for the MF and MPN results) or would result in different water quality classifications for the same sample, based on the State of Connecticut SS standard for recreational bathing water (Table 2). Eight of these 13 samples (5.8% of the total test samples) had statistically different test results between methods. Although the test results were statistically different for these eight samples, in five cases the disagreement made no difference in the determination of acceptability of these bathing areas for recreational use. During the positive Quanti-Tray well confirmation process, two samples with result disagreement, 31023 and 32222 (both marine water), were found to contain a number of false-positive wells. The false fluorescence reactions of these additional positive wells resulted in a higher MPN result for these two samples and caused the disparity between their MPN and MF results. Nevertheless, this discrepancy made no difference in their reporting status. They both would be considered acceptable for recreational use. The microbiological quality of the remaining samples that had result disagreement (31740, 31742, and 31776) varies depending on which test method result is used to make the determination. In all three cases, the MPN result was below the cutoff value of 61/100 ml whereas the MF result exceeded it.

The remaining five samples (Table 2) agreed statistically, but depending on which test method result is used, their quality status was classified differently. Three of these samples (29058, 31773, and 31775) had a lower MPN result, compared with the

TABLE 2. Effect of variable test results (MF versus MPN[a]) on final water quality determination

Sample no.	Source	MF result[b]	MPN result[b]	MF-MPN result agreement[c]	Sample quality (MF/MPN)[d]
29058	Fresh	110	53	Y	NA/A
29061	Fresh	220	64	N	NA/NA
30624	Fresh	40	87	Y	A/NA
31016	Fresh	60	10	N	A/A
31023	Marine	<10	42	N	A/A
31737	Marine	40	75	N	A/A
31740	Fresh	280	53	N	NA/A
31742	Fresh	70	10	N	NA/A
31773	Fresh	100	53	Y	NA/A
31775	Marine	120	53	Y	NA/A
31776	Marine	150	53	N	NA/A
32209	Marine	30	75	Y	A/NA
32222	Marine	<10	42	N	A/A

[a] Enterolert MPN.
[b] Number of enterococci per 100 ml.
[c] Correlation of test method confidence limit ranges. Y, yes; N, no.
[d] Recreational use: A, acceptable; NA, not acceptable.

MF result, and in each case, just one more positive Enterolert well in each of these three samples would have resulted in agreement.

In our study, the false-positive and false-negative rates for Enterolert were found to be 5.1 and 0.4%, respectively. The standard mE MF method evaluated by Levin et al. (13) was found to have noticeably higher rates (false positive, 10.0%; false negative, 11.7%), and several state public health department laboratories have reported similar rates on the basis of observations of routine MF testing of recreational waters (unpublished correspondence). Compared with the MF procedure, we have found that Enterolert more effectively and accurately recovers enterococci from recreational bathing waters. This may be due to various factors such as the ability of Enterolert to repair and recover stressed cells, potential problems associated with procedures using MFs, and test reading objectivity. For example, in our study we also tested five river water samples obtained near the effluent of treated sewage. One of these samples gave significantly different test results between methods (MF, <10; Enterolert, >2,005), and because of the proximity to treated sewage effluent, this sample most likely contained chlorine-stressed enterococci. These preliminary results suggest that Enterolert may be better at recovering injured enterococci.

When membrane filter procedures are used, inaccurate counts could result from variations in gridded MF quality (i.e., toxic and growth-promoting substances, variable pore sizes, etc.) (12) and obstruction of the agar surface by sample particulate matter. Also, in samples containing high levels of suspended solids, growth-inhibiting chloro-organic complexes may become trapped on membrane surfaces (14).

On esculin-iron agar used in the MF confirmation step, the positive reaction intensity may vary depending on colony density and size, and counting errors could result from the subjectivity of this reaction. The Enterolert well positive reaction is clearer (any fluorescence is considered a positive reaction), and less training and experience are required to read the test, and so therefore the potential for errors is decreased. In our study, mean test result values for all samples tested were lower for the MPN method (34.0 versus 40.0 enterococci per 100 ml for MF), as was also the case when the sample categories were looked at separately (marine and freshwater). These results may in fact be reflecting the lower false-positive rate of Enterolert compared with the MF method.

Overall quality control testing requirements for Enterolert are fewer (one-time testing of the single system) compared with the MF method (filters, plate agar batch testing, and daily equipment and supply maintenance). Also, Enterolert reagent shelf life and storage conditions are preferable (1 year from manufacture at room temperature) to those for MF agar plate media (2 weeks refrigerated).

Time studies indicate that Enterolert requires significantly less time per sample for setup, reading, and recording of results. These time savings, in addition to a reduction in the incubation time required (24 versus 48 h for the MF procedure), decrease turnaround time significantly and allow public health officials to more rapidly assess the quality of recreational bathing areas and take appropriate action if required. In addition, the high specificity of Enterolert decreases the frequency of unnecessary closure of bathing areas based on false-positive results. Using Enterolert to screen the recreational bathing areas tested in this study would have resulted in an overall 2.9% decrease in closure rates compared with using the MF method. These issues are of primary concern to public health authorities involved in maintaining and monitoring recreational bathing areas. If the enterococcal levels in a public recreational bathing area are found to exceed the standards for acceptability, the bathing area is kept closed until the microbiological quality of the water is shown to be acceptable through repeat sampling and testing. Closure time translates into significant revenue losses, especially during peak season. Our study demonstrates that using Enterolert would allow these areas to be opened for use more quickly.

We have found that Enterolert can provide results sooner than the membrane filter technique with an increase in sensitivity and specificity. The resultant decrease in turnaround time translates into cost savings and allows officials to more quickly respond to potentially hazardous public health situations. These characteristics make this methodology a practical choice when testing recreational bathing waters for microbiological quality.

REFERENCES

1. **American Public Health Association.** 1995. Standard methods for the examination of water and wastewater, 19th ed., section 9230C. American Public Health Association, Washington, D.C.
2. **Berg, J. D., and L. Fiksdal.** 1988. Rapid detection of total and fecal coliforms in water by enzymatic hydrolysis of 4-methyl-umbelliferone-beta-D-galactoside. Appl. Environ. Microbiol. **54:**2118–2122.
3. **Bordner, R., J. A. Winter, and P. V. Scarpino (ed.).** 1978. Microbiological methods for monitoring the environment: water and wastes. EPA-600/8-78-017. U.S. Environmental Protection Agency, Cincinnati.
4. **Cabelli, V. J.** 1983. Health effects criteria for marine recreational waters. EPA-600/1-80-031. U.S. Environmental Protection Agency, Research Park Triangle, N.C.
5. **Cabelli, V. J.** 1989. Swimming associated illness and recreational water quality criteria. Water Sci. Technol. **21:**13–21.
6. **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.
7. **Dufour, A. P.** 1984. Health effects criteria for fresh recreational waters. EPA-600/1-84-004. U.S. Environmental Protection Agency, Research Park Triangle, N.C.
8. **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.
9. **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.
10. **Facklam, R. R., and D. F. Sahm.** 1995. Enterococci, p. 308–314. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 6th ed. American Society for Microbiology, Washington, D.C.
11. **Fiksdal, L., M. Pommepuy, M.-P. Caprais, and I. Midttun.** 1994. Monitoring of fecal pollution in coastal waters by use of rapid enzymatic techniques. Appl. Environ. Microbiol. **60:**1581–1584.
12. **Geldreich, E.** 1975. Performance variability of membrane filter procedures, p. 12–19. *In* Symposium on the recovery of indicator organisms employing membrane filters. EPA-600/9-77-024. U.S. Environmental Protection Agency, Cincinnati.
13. **Levin, M. A., J. R. Fischer, and V. J. Cabelli.** 1975. Membrane filter technique for enumeration of enterococci in marine waters. Appl. Microbiol. **30:**66–71.
14. **McKee, J. E., and R. T. McLaughlin.** 1958. Application of molecular filter techniques to the bacterial assay of sewage. II. Experimental results for settled sewage. Sewage Ind. Wastes **30:**129–137.
15. **Mundt, J. O.** 1986. Enterococci, p. 1063–1065. *In* P. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. Williams & Wilkins, Baltimore.
16. **Palmer, C. J., Y.-L. Tsai, A. L. Lang, and L. R. Sangermano.** 1993. Evaluation of Colilert-Marine Water for detection of total coliforms and *Escherichia coli* in the marine environment. Appl. Environ. Microbiol. **59:**786–790.
17. **U.S. Environmental Protection Agency.** 1985. Test methods for *Escherichia coli* and enterococci in water by the membrane filter procedure. EPA-600/4-85/076. U.S. Environmental Protection Agency, Cincinnati.
18. **U.S. Environmental Protection Agency.** 1986. Ambient water quality criteria for bacteria—1986. EPA-440/5-84-002. U.S. Environmental Protection Agency, Cincinnati.