APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1979, p. 928-931
0099-2240/79/05-0928/04$02.00/0
Vol. 37, No. 5

Comparison of *Limulus* Assay, Standard Plate Count, and Total Coliform Count for Microbiological Assessment of Renovated Wastewater

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Received for publication 16 February 1979

The *Limulus* endotoxin assay was compared to the standard plate count and total coliform count for assessment of the bacteriological quality of reclaimed wastewater. A total of 48 water samples from an advanced waste treatment plant in Dallas, Tex. were examined by the three techniques. *Limulus* assays were technically simpler to perform and provided results much sooner than conventional culture methods. However, the endotoxin values did not correlate extremely well with determinations of viable bacterial numbers. This lack of correlation may have been due to alterations in the normal ratio of viable gram-negative cells to endotoxin caused by water reclamation procedures.

Efforts by various investigators continue toward the development of more rapid, specific methods of assessing water quality (2, 12, 14). These methods are intended to augment or replace the more classic techniques of standard plate count and total coliform counts of water, which require more than 24 h for results (1). More rapid methods for assessment of water quality could prove immediately useful in several areas of public health and environmental microbiology.

The *Limulus* in vitro endotoxin assay has been demonstrated to have possible utility for the examination of potential and actual drinking waters (3, 7). The measurement of total or bound endotoxin content of a fluid may be used as a means of quantifying the number of gram-negative bacteria present (5). The *Limulus* assay is a rapid technique (total test time of less than 2 h) that does not require specialized equipment or facilities for performance. Therefore, the *Limulus* test is potentially useful for prompt on-site determinations of water or wastewater effluent quality.

MATERIALS AND METHODS

Water samples. Water samples were supplied to our laboratory for testing by the Henry J. Graeser Environmental Research and Training Facility, an advanced wastewater treatment pilot plant located in Dallas, Tex. The facility was operated in a mode to produce a high quality wastewater effluent, which included primary treatment followed by activated sludge to nitrify and partially denitrify, mixed media filtration and carbon adsorption (two columns in series) with three disinfection processes, ozone, ultraviolet irradiation, and chlorination provided as needed. Processed water samples were collected in sterile, pyrogen-free plastic test tubes (Falcon) at the Graeser facility, immediately cooled to 4 to 8°C in wet ice, and shipped by aircraft or by bus to our laboratory in San Antonio, Tex. Samples were then either processed immediately or refrigerated until tested on the following day.

Standard plate counts. Standard plate counts were performed by using serial dilutions of the water samples in sterile buffered water, followed by preparation of pour plates, using standard plate count agar in replicates of three (1).

Total coliform counts. A standard total coliform count was performed by the membrane filtration technique (1). A 50-ml portion of the water sample was passed through a 0.45-μm membrane filter. The filter was then removed and aseptically placed onto a plastic petri dish (60 by 15 mm) containing m-Endo agar LES (Difco Laboratories). Endo agar plates were examined, and the number of coliform colonies was determined after 24 h of incubation at 35°C. Results were expressed as total coliforms per 100 ml of water.

Performance of *Limulus* assay. *Limulus* assays were performed by methods previously described (7). For determinations of total endotoxin content, tests were performed by reacting dilutions of water sample (prepared in sterile, pyrogen-free water) with a commercial preparation of *Limulus* ameobocyte lysate (Difco Pyrotest). Free endotoxin levels of water samples were determined by *Limulus* assay of the water after passage through a 0.22-μm membrane filter (10). The amount of bound endotoxin was calculated by subtracting the amount of free endotoxin from the amount of total endotoxin.

The endotoxin sensitivity of Difco Pyrotest lots varied slightly, but generally as little as 0.0625 ng of

purified *Escherichia coli* endotoxin per ml could be reliably detected. Actual tests were performed by adding 0.2 ml of the diluted water sample directly to the single test vial containing lyophilized Pyrotest. All assays were incubated for 70 to 90 min at 37°C. After incubation, the presence of a gel or a marked increase in viscosity and turbidity was considered a positive test for endotoxin (8). Quantification of endotoxin was accomplished based on the dilution of the water sample and the sensitivity of the particular Pyrotest lot. The endotoxin concentrations were reported in endotoxin equivalents (11).

Statistical methods. The co-relatedness of measurements of total, bound, and free endotoxin with standard plate counts and total coliform counts were determined by calculation of the respective correlation coefficients (r) (13).

RESULTS

A total of 48 water samples were examined by the three test methods. Standard plate counts varied from less than 10 organisms per ml to 5.5 $\times 10^4$/ml. Total coliform counts ranged from less than 2×10^0 to 1.4×10^6/100 ml. The total endotoxin content of the unfiltered water samples ranged from 6 to 600 ng of endotoxin equivalents per ml; free endotoxin levels (filtered water) ranged from 3 to 480 ng of endotoxin equivalents per ml, and the bound endotoxin content ranged from 0 to 250 ng of endotoxin equivalents per ml. The data (Fig. 1) compare the relationship between total endotoxin content and total number of bacteria determined by standard plate count. The correlation coefficient was determined to be 0.726. Figure 2 examines the relationship of bound endotoxin content to standard plate count ($r = 0.736$). Figure 3 compares free endotoxin content with the standard plate count ($r = 0.620$). Figure 4 compares total endotoxin content with total coliform count ($r = 0.822$), and Fig. 5 compares bound endotoxin content with total coliform count ($r = 0.472$).

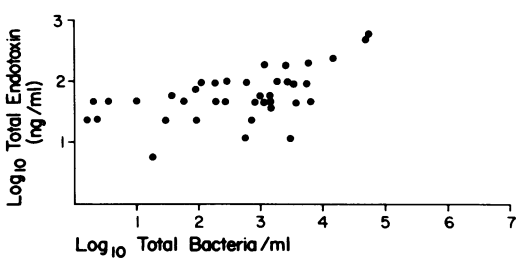

FIG. 1. *Relationship between total endotoxin and standard plate count (r = 0.726).*

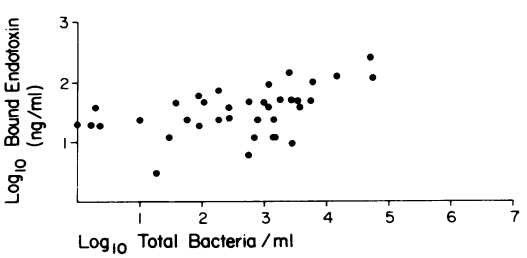

FIG. 2. *Relationship between bound endotoxin and standard plate count (r = 0.736).*

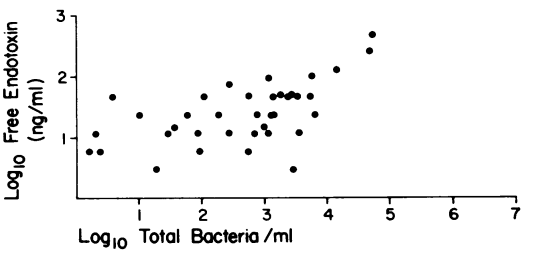

FIG. 3. *Relationship between free endotoxin and standard plate count (r = 0.620).*

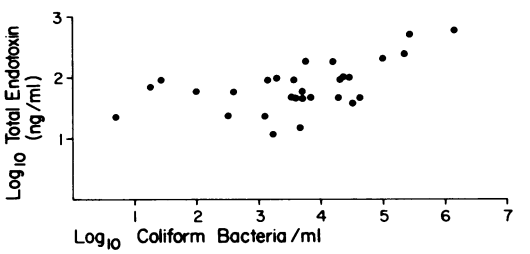

FIG. 4. *Relationship between total endotoxin and total coliform count (r = 0.822).*

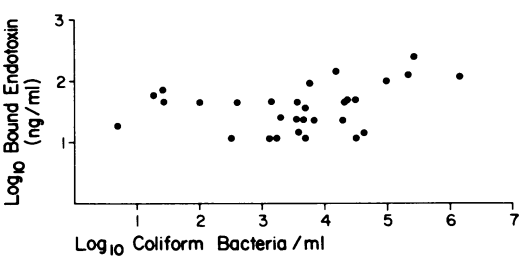

FIG. 5. *Relationship between bound endotoxin and total coliform count (r = 0.472).*

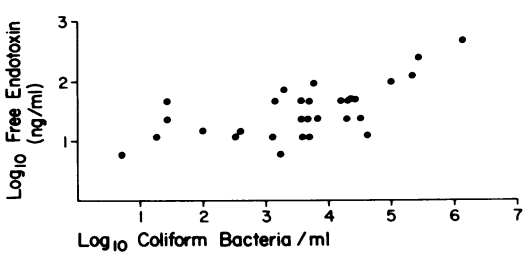

FIG. 6. *Relationship between free endotoxin and total coliform count (r = 0.525).*

Figure 6 relates the free endotoxin content to the total coliform count ($r = 0.525$).

DISCUSSION

A previous preliminary investigation demonstrated the feasibility of testing water samples by the *Limulus* assay for the presence of endotoxin due to gram-negative bacteria (7). The *Limulus* assay appears to be a rapid, simple, relatively inexpensive technique for detecting bacterial endotoxins in water or wastewater. A technique of this type might prove useful in the rapid determination of levels of coliform bacteria in various kinds of water (3). However, in this study using reclaimed wastewater, the results of *Limulus* assays did not correlate extremely well with two established techniques for assessing water quality, i.e., the standard plate count and total coliform count. Levels of total, bound, or free endotoxin activities seemed unable to reliably predict the microbial content of the water samples examined if chlorination, ozone, or ultraviolet treatments had been performed.

The *Limulus* assay has previously been successfully used to obtain approximate quantification of viable bacteria in stream water (3), seawater (15), urine (5, 6), parenteral fluids (9), and even ground beef (4). The explanation for this lies in the relatively constant amount of bound endotoxin associated with the cell walls of viable, gram-negative bacteria. Although free endotoxin accumulation occurs during growth in a liquid environment, its concentration appears to parallel that of bound or cell-associated endotoxin (10). It has also been shown that antibiotic-killed gram-negative bacteria continue to possess endotoxin activity detectable by the *Limulus* assay (5).

The relative lack of correlation between endotoxin levels and densities of viable gram-negative bacteria observed in the present study must in large part be due to detection of varying combinations of viable and nonviable bacterial cells by the *Limulus* assay. Treatments of the effluent such as chlorination or exposure to UV irradiation or ozone will kill a majority of the bacterial population present but not necessarily destroy or detoxify bacterial endotoxins. When 24 samples, obtained from the Graeser facility before these end process treatments, were examined, the correlations between the standard plate count and total endotoxin ($r = 0.945$), standard plate count and free endotoxin ($r = 0.932$), and total coliform and free endotoxin ($r = 0.939$) were very encouraging. However, the correlations between standard plate counts and bound endotoxin ($r = 0.745$), total coliforms and total endotoxin ($r = 0.822$), and total coliforms and bound endotoxin ($r = 0.419$) were less predictive.

In the study by Evans et al. (3), better correlations between endotoxin content and bacterial numbers were achieved by use of the spectrophotometric modification of the *Limulus* assay than by the clot formation endpoint method, as utilized in the current study. Evans et al. found that the bound endotoxin component (which was most predictive of bacterial numbers) was decreased after chlorination procedures. However, it has also been previously demonstrated that agitation of gram-negative bacteria in a liquid environment serves to release additional amounts of cell-associated endotoxin (10). Thus, we reason that the poor correlation between endotoxin content and densities of viable microorganisms determined in this study results from a combination of partial destruction of gram-negative bacteria and varying degrees of solubilization of cell-associated endotoxin. Therefore, the endotoxin content of highly treated wastewater effluent, unlike stream water (3) or seawater (15), probably reflects the remnants of preexistent bacterial growth rather than continued proliferation of gram-negative microorganisms. However, an additional possibility that must be considered is that neither the total coliform count nor the standard plate count necessarily reflects an accurate measure of the total gram-negative bacterial population. There may be other gram-negative bacteria with more complex nutritional requirements or different temperature or atmospheric requirements (such as anaerobic bacteria) that contribute to the endotoxin content, as measured by the *Limulus* assay. Therefore, it is possible that certain of these proliferate in reclaimed wastewater and are reflected in the endotoxin measurements, but not detected by the two standard methods of determining water quality. Including media and incubation conditions appropriate for these might have improved the correlation between viable gram-negative bacteria and endotoxin.

The significance of bacterial endotoxins, per se, in potential drinking water remains unclear. Experiments to determine the possible pyrogenic effects of consuming water with varying endotoxin content are being conducted on experimental animals. For the moment, it does not seem likely that the detection of bacterial endotoxin by the *Limulus* assay is an adequate predictor of the sanitary quality of reclaimed or highly treated wastewater.

ACKNOWLEDGMENTS

This study was supported in part by grant R-804420010 from the United States Environmental Protection Agency.

We thank the staff of the Henry J. Graeser Environmental Research Center for their assistance during the course of the investigation.

LITERATURE CITED

1. **American Public Health Association.** 1971. Standard methods for the examination of water and waste water, 13th ed. American Public Health Association, New York.
2. **Bachrach, U., and Z. Bachrach.** 1974. Radiometric method for the detection of coliform organisms in water. Appl. Microbiol. **28**:169–171.
3. **Evans, T. M., J. E. Schillinger, and D. G. Stuart.** 1978. Rapid determination of bacteriological water quality by using *Limulus* lysate. Appl. Environ. Microbiol. **35**:376–382.
4. **Jay, J. M.** 1977. The *Limulus* lysate endotoxin assay as a test of microbial quality of ground beef. J. Appl. Bacteriol. **43**:99–109.
5. **Jorgensen, J. H., H. F. Carvajal, B. E. Chipps, and R. F. Smith.** 1973. Rapid detection of gram-negative bacteriuria by use of the *Limulus* endotoxin assay. Appl. Microbiol. **26**:38–42.
6. **Jorgensen, J. H., and P. M. Jones.** 1975. Comparative evaluation of the Limulus assay and the direct Gram stain for detection of significant bacteriuria. Am. J. Clin. Pathol. **63**:142–148.
7. **Jorgensen, J. H., J. C. Lee, and H. R. Pahren.** 1976. Rapid detection of bacterial endotoxins in drinking water and renovated wastewater. Appl. Environ. Microbiol. **32**:347–351.
8. **Jorgensen, J. H., and R. F. Smith.** 1973. Preparation, sensitivity, and specificity of *Limulus* lysate for endotoxin assay. Appl. Microbiol. **26**:43–48.
9. **Jorgensen, J. H., and R. F. Smith.** 1973. Rapid detection of contaminated intravenous fluids using the *Limulus* in vitro endotoxin assay. Appl. Microbiol. **26**:521–524.
10. **Jorgensen, J. H., and R. F. Smith.** 1974. Measurement of bound and free endotoxin by the Limulus assay. Proc. Soc. Exp. Biol. Med. **146**:1024–1031.
11. **Moody, E. E. M., M. D. Trousdale, J. H. Jorgensen, and A. Shelokov.** 1975. Bacteriophages and endotoxin in licensed live-virus vaccines. J. Infect. Dis. **131**:588–591.
12. **Newman, J. S., and R. T. O'Brien.** 1975. Gas chromatographic presumptive test for coliform bacteria in water. Appl. Microbiol. **30**:547–552.
13. **Steel, R. G. D., and J. H. Torrie (ed.).** 1960. Principles and procedures of statistics. McGraw-Hill Book Co., New York.
14. **Warren, L. S., R. E. Benoit, and J. A. Jessee.** 1978. Rapid enumeration of fecal coliforms in water by a colorimetric β-galactosidase assay. Appl. Environ. Microbiol. **35**:136–141.
15. **Watson, S. W., T. J. Novitsky, H. L. Quinby, and F. W. Valois.** 1977. Determination of bacterial number and biomass in the marine environment. Appl. Environ. Microbiol. **33**:940–946.