

Rapid Determination of Bacteriological Water Quality by Using *Limulus* Lysate

T. M. EVANS,† J. E. SCHILLINGER,* AND D. G. STUART

Department of Microbiology, Montana State University, Bozeman, Montana 59717

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The *Limulus* lysate assay was used to measure the endotoxin content in stream water and was found to reflect the degree of bacterial contamination as measured by coliform, enteric, gram-negative, and heterotrophic bacteria. The firm-clot method was found to be a less sensitive and reproducible technique for the detection of endotoxin than was the spectrophotometric modification of the *Limulus* lysate assay. Bound endotoxin, as determined by the spectrophotometric modification of the *Limulus* lysate assay, was found to be a better measure of the endotoxin associated with bacterial cells than was total endotoxin.

At present, bacteriological tests for indicator organisms are the only accepted means for assessing the sanitary quality of water supplies. An important limitation of these tests is the 24 or 48 h required to perform them and obtain results. A rapid and simple test of bacterial water quality would have many applications, especially in disaster situations, such as floods or hurricanes, and in instances of treatment system failure.

Several investigators (3, 5, 15) have suggested that the *Limulus* lysate assay for endotoxin may be a useful technique for rapidly determining bacterial quality of water. This technique requires only 2 h to perform and detects the lipopolysaccharide or endotoxin portion of gram-negative bacterial cell envelopes. Most, if not all, gram-negative bacteria possess endotoxins (2, 12), whereas gram-positive bacteria do not give a positive *Limulus* lysate test (13). One investigator (4) did find that a viral ribonucleic acid gave a positive test in approximately the same concentrations as endotoxin.

The *Limulus* lysate assay was developed by Levin and Bang (10), who observed that the in vitro coagulation of *L. polyphemus* amoebocytes was mediated by gram-negative bacterial endotoxin. Refinements of the assay have shown that as little as 1 pg of endotoxin per ml can be detected (17, 19).

The present study was undertaken to test the applicability of the *Limulus* lysate assay as a rapid test of water quality and to correlate the amount of endotoxin in water with other measures of bacterial water quality.

MATERIALS AND METHODS

Description of sampling sites. The East Gallatin drainage system (Fig. 1) starts out south of Bozeman, Mont., as high, pristine mountain streams (sites EF1, H3, and M3), which traverse the valley floor passing through agricultural (sites M4, H4, and H5) and urban areas (site M5). A mixture of primary and secondary sewage effluent enters the East Gallatin River about 13.3 km (0.8 mile) above site EG5. Site EG5a is located about 4.8 km (3.0 miles) downstream from the sewage outfall (OF2). The range of sites provided samples varying from clean snowmelt waters to those impacted by sewage pollution.

Bacterial enumeration. Grab samples for bacterial analysis were collected in 2-liter sterile Nalgene bottles. For sites OF2 and EG5, 2 ml of a 10% solution of sodium thiosulfate was added to neutralize any residual chlorine that may have been present. Samples for endotoxin analysis were collected in screw-cap culture tubes rendered pyrogen free by baking at 180°C for 4 h. Samples for endotoxin and bacterial analyses were collected simultaneously and held on ice until tested. All samples were tested within 6 h of collection.

Total coliform populations were enumerated by the membrane filter procedure on m Endo-MF agar (Difco Laboratories, Detroit, Mich.) (1).

Heterotrophic bacteria were enumerated by the spread-plate technique employing the casein-peptone-starch medium (CPS) of Stark and McCoy (16). After the plates were inverted and dried for 24 h at room temperature, they were stored at 4°C for no longer than 48 h before use. Dilutions were made in 9-ml blanks of phosphate buffer (1) supplemented with 0.1% peptone, and inoculation was done by the spread-plate method. The plates were inverted, incubated for 7 days at 20°C, and counted with the aid of a colony counter (New Brunswick Scientific Co., New Brunswick, N.J.).

Counts of enteric bacteria were obtained with Tergitol 7 agar (Difco) supplemented with 1 ml of a 1% solution of triphenyltetrazolium chloride after auto-

† Present address: Department of Microbiology, University of New Hampshire, Durham, NH 03824.

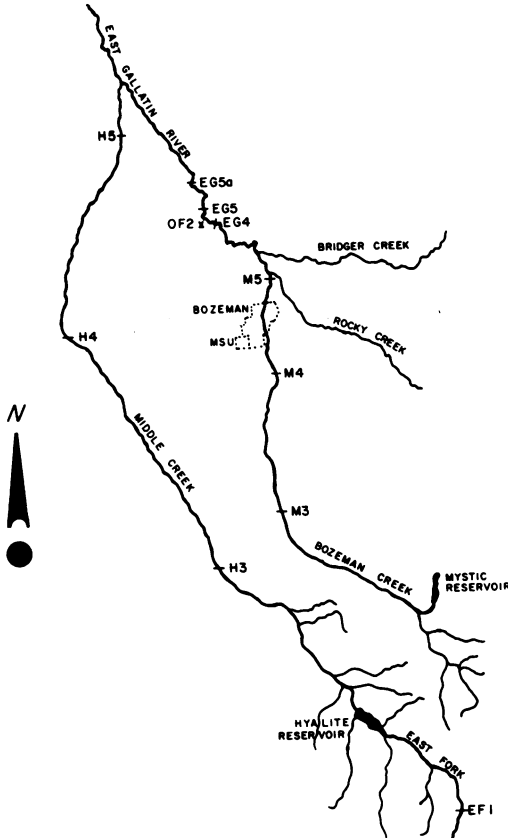


FIG. 1. Sampling sites for bacterial and endotoxin analysis.

claving. Either the spread-plate technique or the membrane filtration procedure was used, and the inverted plates were incubated for 48 h at 20°C.

Gram-negative bacterial counts were obtained by membrane filtration, when endotoxin was determined by the firm-clot procedure, and by the spread-plate procedure, when endotoxin was determined by the spectrophotometric modification of the *Limulus* lysate assay. The membrane filtration medium was a modification of Holding medium (6) and consisted of a tryptic soy agar (Difco) base supplemented with 0.3% yeast extract, 0.5% glucose, and 2 µg of crystal violet per ml. The selectivity of this medium was determined by inhibition testing of various concentrations of the selective agent with 12 gram-negative and 9 gram-positive bacteria. In addition, 20 colonies from water sample plates were picked, transferred to tryptic soy broth (Difco), incubated for 18 h at 35°C, and Gram stained. After it became apparent that crystal violet at this concentration was inhibitory to *Escherichia coli*, a new selective spread-plate medium was developed. Ethyl violet (Matheson, Coleman, and Bell, Norwood, Ohio) at a concentration of 1.25 µg/ml has been reported to be a satisfactory selective agent for gram-negative bacteria (11). The selectivity of ethyl violet in a CPS agar base was tested as before. Since

not all of the gram-positive bacteria tested were inhibited, penicillin (Eli Lilly & Co., Indianapolis, Ind.) was incorporated into the medium. Selectivity of ethyl violet in conjunction with penicillin was tested as before. The final concentration of ethyl violet and penicillin found to be effective was 1.25 µg and 10 U per ml, respectively. The sensitivity of *E. coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Shigella flexneri* to ethyl violet-penicillin-casein-peptone-starch medium (EVP) was tested by diluting and plating 24-h cultures, grown in tryptic soy broth, onto EVP, CPS, and standard methods agar and incubating at 20°C for 7 days. Water samples were plated as described above on the EVP medium, and plates were inverted and incubated for 7 days at 20°C.

Endotoxin determination. To ensure against contamination and false reactions, all glassware was rendered pyrogen free by baking at 180°C for 4 h. Pyrogen-free distilled water (Cutler Laboratories, Inc., Berkeley, Calif.) was dispensed into pyrogen-free 250-ml flasks, covered with aluminum foil, and autoclaved for 3 h on the day it was used.

Limulus lysate was obtained in lyophilized form (Associates of Cape Cod, Woods Hole, Mass.). It was reconstituted immediately before use by the addition of 7.5 ml of pyrogen-free water and stored on ice during use. The lysate solution was clarified by centrifuging at 7,680 × g. Endotoxin preparations used in initial experiments included Westphal phenol extracts of *E. coli* 0111:B4 (Difco) and *Klebsiella* sp. ATCC-12833 (FDA). For the spectrophotometric determinations, *E. coli* 180-10 endotoxin (Associates of Cape Cod) was used. All were reconstituted with pyrogen-free distilled water to a stock solution concentration of 100 ng/ml. Twofold serial dilutions of the stock solution were made using a 0.1-ml Eppendorf pipette to obtain standard solutions ranging from 1 ng to 1 µg per ml. The disposable tips for the pipette were found to be pyrogen free as were the sterile polystyrene tubes (12 by 75 mm; Falcon Plastics, Oxnard, Calif.) used for dilutions and reaction tubes. Control tubes containing 0.2 ml of lysate and tubes containing 0.1 ml of pyrogen-free water plus 0.1 ml of lysate were run routinely.

Initial experiments were performed by the firm-clot method of Jorgensen and Smith (9). Twofold serial dilutions of standard endotoxin or the water sample (0.1-ml volumes) were incubated with lysate (0.1 ml) for 1 h at 37°C in a circulating water bath. At the end of the incubation period, the tubes were slowly removed from the water bath and gently inverted 180°. The end point of the test was the highest dilution that clotted the lysate to a degree that the clot did not break when inverted. The standard endotoxin end point dilution was used to obtain the sample endotoxin concentration.

Other experiments involved the spectrophotometric determination of endotoxin concentrations. Stanley Watson (Woods Hole Oceanographic Institution) modified the *Limulus* lysate assay so that the reaction of lysate and endotoxin formed a turbid suspension instead of a firm clot. Two modifications were made in that procedure as follows: (i) pyrogen-free 0.1% NaCl instead of 3.0% NaCl was used as a diluent, and (ii) 0.5 ml of sample and 0.1 ml of lysate were used instead of

1.0 and 0.2 ml, respectively. The standard endotoxin dilutions and the threefold sample serial dilutions were made to result in 0.5-ml volumes to which 0.1-ml portions of lysate were added. All tubes were then mixed on a Vortex shaker and incubated at 37°C in a water bath for exactly 1 h. After the incubation period, the tubes were removed from the water bath, mixed thoroughly, and poured into microcuvettes (Coleman Instruments, Maywood, Ill.) having a 1-cm light path. The absorbancy at 360 nm was immediately determined on a spectrophotometer (Varian Techtron model 635). The instrument was zeroed with pyrogen-free 0.1% NaCl. The turbidity of the samples slowly increased with time, but staggering the addition of lysate to sets of samples and reading each tube in a set within 5 min (after 1 h) made the increase negligible.

A standard curve for the *Limulus* lysate assay was made by plotting absorbance at 360 nm against endotoxin concentration. Figure 2 shows a typical standard curve for endotoxin concentrations.

The amount of free endotoxin in the water samples was determined by testing the supernatant fluid obtained by centrifuging the water samples for 10 min in pyrogen-free glass centrifuge tubes at $12,100 \times g$. The amount of bound endotoxin in the sample was found by subtracting the concentration of free endotoxin from total endotoxin.

RESULTS

Field experiments involved collection of samples from selected sites on 3 different days. In preliminary experiments with the firm-clot procedure, bacteria were enumerated by the membrane filtration technique. The correlation coefficients listed in Table 1 indicate a better correlation between bacterial groups when they were enumerated by the spread-plate technique. As a result, enteric, gram-negative, and heterotrophic bacteria were enumerated by the spread-plate technique in later experiments involving the spectrophotometric method of the *Limulus* lysate assay. The crystal violet medium showed

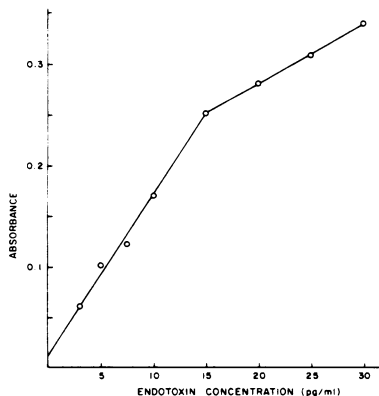


FIG. 2. Standard curve for spectrophotometric endotoxin assay.

only 70% selectivity for gram-negative bacteria and also inhibited *E. coli*. The EVP medium was not inhibitory to the bacteria tested and was nearly 100% selective for gram-negative bacteria. The recovery (Table 2) of gram-negative bacteria on EVP medium was not significantly different (as tested with the *t* statistic, $\alpha = 0.05$) from the recovery on CPS and standard methods agar.

The bacteriological and bound endotoxin profiles from two experiments are presented in Fig. 3 and 4. Endotoxin concentrations were determined using the spectrophotometric technique. Except for site EG4 on 7/3/75 (Fig. 3), each of the bacterial groups closely followed the bound endotoxin trends. This was true over the entire range of contamination from the very low bound endotoxin levels at EF1 (0.07 ng/ml) to the high levels observed in the sewage effluent (85.0 ng/ml). Trends similar to those depicted in Fig. 3 and 4 were observed for bacteria and total endotoxin as determined by the firm-clot technique and by the spectrophotometric method. However, the correlation coefficients using the clot procedure were lower than those obtained when using the spectrophotometric technique (Table 3). All the correlations were significant at the 1% level. The comparison may be clouded by having used membrane filters during the

TABLE 1. Correlation coefficients for bacterial group enumeration comparisons using log-transformed data

Variables	Method of bacteria enumeration	
	Membrane filtration	Spread plate
Enterics vs coliforms	0.878	0.968 ^a
Gram negatives vs coliforms	0.748	0.951 ^a
Heterotrophs vs coliforms	ND ^b	0.907 ^a
Gram negatives vs enterics	0.870	0.987
Heterotrophs vs enterics	ND	0.943
Heterotrophs vs gram negatives	ND	0.959

^a Coliforms enumerated by the membrane filtration technique.

^b ND, Not determined.

TABLE 2. Recovery of gram-negative bacteria on complex and selective media

Bacterium	No. of bacteria per ml ^a		
	EVP	CPS	SMA ^b
<i>E. coli</i>	72×10^7	73×10^7	67×10^7
<i>K. pneumoniae</i>	40×10^6	33×10^6	41×10^6
<i>E. aerogenes</i>	59×10^7	58×10^7	65×10^7
<i>S. flexneri</i>	78×10^7	90×10^7	79×10^7

^a Geometric mean of five replicates.

^b SMA, Standard methods agar.

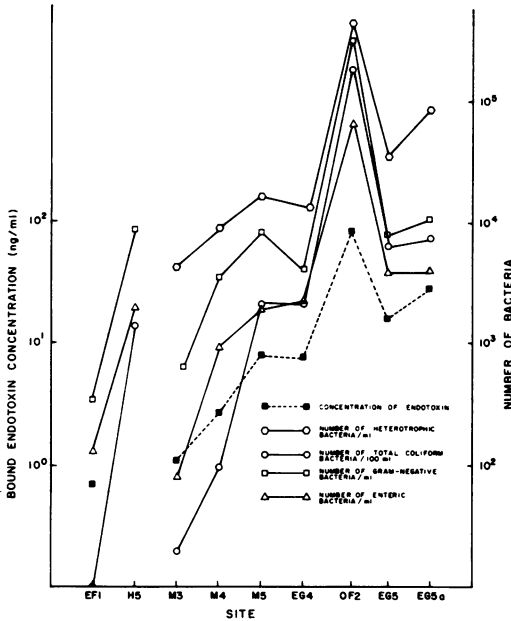


FIG. 3. Bacterial and endotoxin profile of the East Gallatin River drainage for sampling dates 7/3/75, 7/7/75, and 7/8/75. Each point represents the geometric mean of five spread-plate replicate samples.

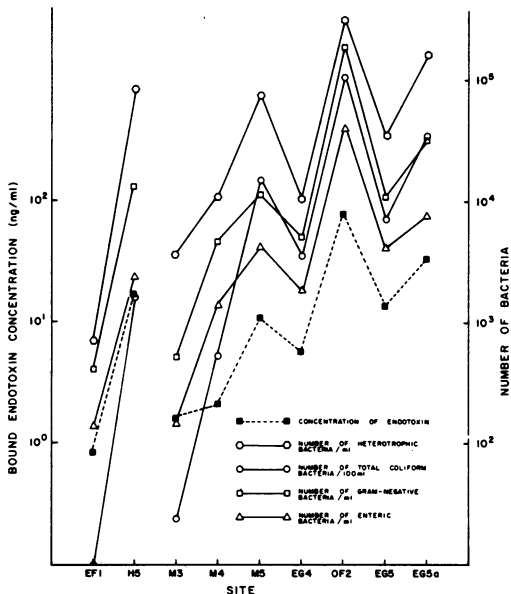


FIG. 4. Bacterial and endotoxin profile of the East Gallatin River drainage for sampling dates 7/9/75, 7/10/75, and 7/14/75. Each point represents the geometric mean of five spread-plate replicate samples.

firm-clot experiments, but total coliforms versus total endotoxin correlation coefficients still showed improvement with the spectropho-

TABLE 3. Correlation coefficients between endotoxin and bacterial groups, significant at the 1% level with log-transformed data

Variables	Method of endotoxin quantification	
	Firm clot	Spectrophotometric
Coliforms vs total endotoxin . . .	0.764	0.829
Enterics vs total endotoxin . . .	0.642	0.905
Gram negatives vs total endotoxin	0.590	0.887
Heterotrophs vs total endotoxin	ND ^a	0.878
Coliforms vs bound endotoxin . . .	ND	0.907
Enterics vs bound endotoxin . . .	ND	0.946
Gram negatives vs bound endotoxin	ND	0.934
Heterotrophs vs bound endotoxin	ND	0.952
Total endotoxin vs bound endotoxin	ND	0.907

^a ND, Not determined.

metric technique. The higher values for the bound endotoxin correlations demonstrated the superior relationships with this endotoxin fraction.

The spectrophotometrically determined total, free, and bound endotoxin concentration values are presented in Table 4. On the average, the percentages of free and bound endotoxin remained fairly stable from site to site except for the sewage outfall (OF2) and the tap water sample. At these sites, chlorination appeared to have caused a decrease in the bound fraction due to increased destruction of bacterial cells.

Correlations between bacterial groups and endotoxin were modeled with linear regression using logarithmic transformations of the data. Figures 5, 6, and 7 show the regression lines and correlation coefficients for heterotrophic bacteria, gram-negative bacteria, and coliforms versus bound endotoxin. Similar curves were obtained with the enteric bacterial group and with plots of the bacterial groups versus total endotoxin. Table 5 shows the correlation coefficients for both total and bound endotoxins versus bacterial groups and the regression equations. All the regressions were significant at the 1% level as determined by the *t* statistics. Some improvement in the scatter is evident with the bound endotoxin regressions. The regression coefficients are quite similar for the heterotrophic, gram-negative, and enteric bacteria, but lower for the coliform regressions.

DISCUSSION

The firm-clot *Limulus* lysate procedure produced significant correlations between total

endotoxin concentrations and bacterial counts. Improved relationships were obtained using the spectrophotometric technique, probably due to increased precision in the endotoxin standard curve, sample determinations, and the use of the

TABLE 4. Relationships between total, free, and bound endotoxin in the East Gallatin drainage

Date and site	Total LPS ^a (ng/ml)	Free LPS (ng/ml)	Bound LPS (ng/ml)
7/3/75			
EG4	19.00	11.50	7.50
OF2	1,049.00	964.00	85.00
EG5	53.19	37.70	15.49
7/7/75			
M3	2.43	1.35	1.08
M4	5.31	2.67	2.65
M5	11.70	3.70	8.00
7/8/75			
EF1	9.70	9.00	0.70
H5	16.10		
EG5A	40.70	12.00	28.70
7/9/75			
EG4	22.10	15.30	6.80
OF2	252.00	171.00	81.00
EG5	24.30	10.80	13.50
7/10/75			
M3	4.80	3.15	1.65
M4	5.31	3.15	2.16
M5	21.00	9.45	11.55
7/14/75			
EF1	2.64	1.80	0.84
H5	24.60	7.50	17.10
EG5A	48.60	16.20	32.40
Tap ^b	1.19	1.10	0.09

^a LPS, Lipopolysaccharide.

^b Drinking water tap at Montana State University.

spread-plate procedure. The correlations between endotoxin fractions and gram-negative bacteria were improved through the use of EVP medium, which was superior to crystal violet agar in terms of enhanced recovery and selectivity. Further improvement in correlation coefficients (Table 3) and regression lines (Table 5) was obtained using the bound endotoxin fractions. This is reasonable because this fraction is composed mainly of intact bacterial cells and would be expected to correlate more closely with the living bacteria enumerated on the different media. The free endotoxin fraction may be more susceptible to variation as a consequence of varying rates of release of endotoxin by different species and strains of bacteria (14).

The high correlation between bound and total endotoxin (Table 3) and the similar slopes of the regression lines developed for both bound and total endotoxin (Table 5) indicated a reasonable constant proportion of bound endotoxin. Varia-

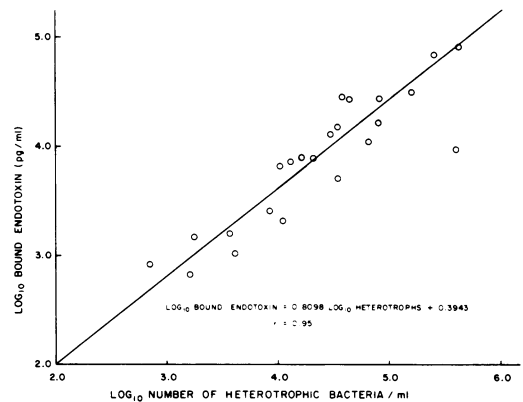


FIG. 5. Relationship between heterotrophic bacteria and bound endotoxin.

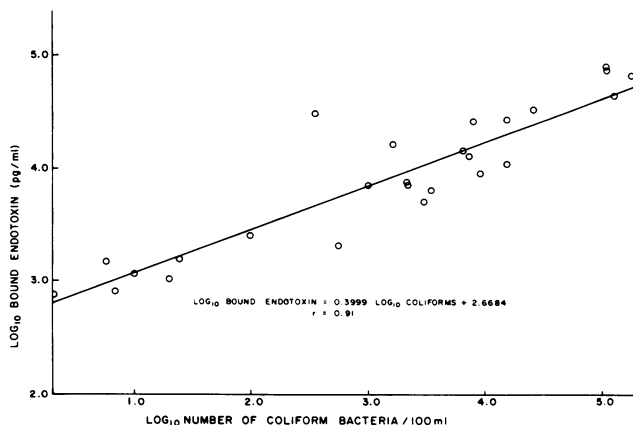


FIG. 6. Relationship between total coliform bacteria and bound endotoxin.

tions in bound and free fractions of endotoxins at different sites may be a reflection of the degrees of injury. The variations were observed to be large in the extreme situations of the chlorinated sewage effluent and tap water sample.

The *Limulus* lysate assay proved to have excellent application to a wide range of conditions and to the different waters tested. One assumption that has been made is that the chemical and physical properties of the waters tested do not affect the endotoxin-clottable protein reaction. Further research is needed to answer this question and to assess the persistence of endotoxin in natural water and in treatment processes. The unlikely high correlations between both bound and total endotoxin and heterotrophic bacteria may be a reflection of the high correlation between gram-negative and heterotrophic bacteria. Also, the regression coefficient for the coliforms versus endotoxin differed from the other bacterial groups, indicating that the coliforms may possess a different proportion of

the total endotoxin. The lower rate of change of bound endotoxin with the number of coliforms may be a consequence of the low proportion of coliforms in the total gram-negative population. The difference might also be caused, in part, by poorer enumeration of the coliform population by the selective procedures used.

Due to the low temperatures and low nutrient conditions in the Gallatin River drainage, the total coliforms correlate well with the degree of contamination and have sanitary significance. Other studies at these sites have shown good correlation between total coliforms and fecal coliforms with correlation coefficients ranging between 0.42 and 0.98. (J. E. Schillinger and D. G. Stuart, Office of Water Research and Technology Completion Report, Montana Univ. Joint Water Resources Research Ctr. report no. 74, 1976). The high positive correlations between the different bacterial groups and total coliforms in this study showed that each bacterial group responded to increases in bacterial contamination; i.e., as contamination increased, the numbers of coliform, enteric, gram-negative, and heterotrophic bacteria increased proportionally. In contrast to the limits of bacterial detection in urine of 1,000 gram-negative bacteria per ml shown by Jorgensen et al. (7), results of this study show good correlation is possible with some waters containing as few as 100 gram-negative bacteria per ml.

The usefulness of the assay in addition to sanitary considerations might be extended to assessing such bacteria as iron bacteria, sulfate-reducing bacteria, and a number of sheathed bacteria in pipes and water mains. These populations are undesirable in terms of corrosion and taste and odor problems and may have their own public health significance.

Additional refinements of the *Limulus* lysate assay are needed to make the procedure amenable to routine water quality testing. Jorgensen

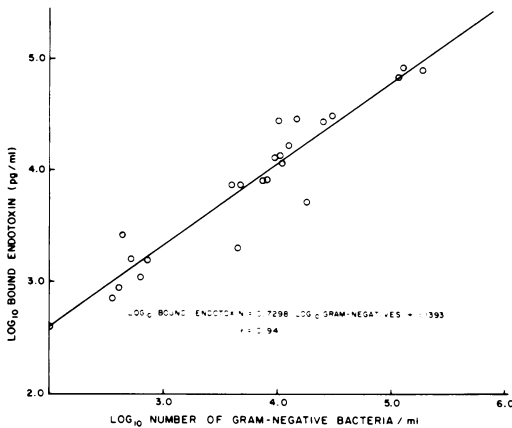


FIG. 7. Relationship between gram-negative bacteria and bound endotoxin.

TABLE 5. Regression equations and correlation coefficients for total endotoxin and bound endotoxin relationships with bacterial counts using log-transformed data

Variables ^a	r ^b	Regression equations
Coliforms vs total endotoxin	0.83	log ₁₀ TE ^c = 0.401 log ₁₀ coliforms + 3.069
Enterics vs total endotoxin	0.91	log ₁₀ TE = 0.802 log ₁₀ enterics + 1.625
Gram negatives vs total endotoxin	0.88	log ₁₀ TE = 0.759 log ₁₀ gram negatives + 1.434
Heterotrophs vs total endotoxin	0.88	log ₁₀ TE = 0.821 log ₁₀ heterotrophs + 0.753
Coliforms vs bound endotoxin	0.91	log ₁₀ BE ^d = 0.400 log ₁₀ coliforms + 2.668
Enterics vs bound endotoxin	0.95	log ₁₀ BE = 0.763 log ₁₀ enterics + 1.351
Gram negatives vs bound endotoxin	0.94	log ₁₀ BE = 0.730 log ₁₀ gram negatives + 1.139
Heterotrophs vs bound endotoxin	0.95	log ₁₀ BE = 0.810 log ₁₀ heterotrophs + 0.394

^a Bacteria enumerated by spread-plate technique, and endotoxin was determined using spectrophotometric procedure.

^b All correlation coefficients were significant at the 1% level.

^c TE, Total endotoxin.

^d BE, Bound endotoxin.

et al. (8) used a commercial *Limulus* lysate kit (Pyrotest, Difco) and found the firm-clot technique to be easily adapted to testing drinking and highly treated waste waters. At least two other such kits are commercially available (Culligan, Inc., Northbrook, Ill., and Microbiological Associates, Bethesda, Md.). A kit utilizing the spectrophotometric procedure (Worthington Biochemical Corp., Freehold, N.J.) is also on the market. The relationships observed in this study hold promise for continued refinements and applications of the *Limulus* lysate assay. The successful development of this rapid test would be a significant advancement for fields like water microbiology and the pharmaceutical industry.

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