APPLIED MICROBIOLOGY, July 1973, p. 38-42

Vol. 26, No. 1
Printed in U.S.A.

Rapid Detection of Gram-Negative Bacteriuria by Use of the *Limulus* Endotoxin Assay

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Received for publication 30 March 1973

The *Limulus* in vitro endotoxin assay was evaluated as a possible method for the prompt detection of significant gram-negative bacteriuria in children. This assay is capable of detecting endotoxin associated with intact cell walls of viable gram-negative bacteria as well as free endotoxin. Quantitative results are obtained following a 1-h incubation of *Limulus* lysate and 10-fold dilutions of otherwise untreated urine. A standard curve of *Limulus* activity and viable cell counts of *Escherichia coli* and *Klebsiella pneumoniae* in urine demonstrated that a positive *Limulus* reaction at a dilution of 1:100 or 1:1,000 indicated a colony count of at least 100,000 bacteria/ml. A positive *Limulus* reaction only from undiluted urine or at a dilution of 1:10 indicated less than 100,000 cells/ml. These experimental observations were confirmed by comparing the *Limulus* test with quantitative plate counts on 209 urine specimens from a mixed pediatric population. These results indicate that the *Limulus* assay is a simple, accurate method for rapid presumptive detection of gram-negative bacteriuria in patients where an immediate diagnosis is needed. This test would also seem promising for screening large patient populations for bacteriuria or for monitoring the effectiveness of treatment of urinary tract infections.

Since Kass (8) first defined the concept of significant bacteriuria, a colony count of more than 100,000 bacteria per ml of urine by quantitative culture has been considered by several investigators (7, 11) to be the best laboratory procedure available for documenting the presence of urinary tract infections. However, facilities for bacteriological processing may not be readily available in all clinics or physicians' offices. Furthermore, a period of 24 to 48 h is usually required for cultivation and characterization of organisms from urine.

A simple, rapid screening test for the diagnosis of significant bacteriuria would seem to be of importance in many situations. The microscope observation of bacteria in a Gram-stained smear of a drop of fresh, uncentrifuged urine has been reported to correlate with significant colony counts in 75 to 95% of cases (8, 16). The use of the phase-contrast microscope has also been proposed to detect bacteria in unstained and uncentrifuged urine (2, 9). Chemical tests for bacteriuria that depend on the presence of bacterial metabolites or certain enzymes in greater amounts than normal in urine have also been proposed. However, measurement of nitrate reductase by the Griess test or tetrazolium reductase may lead to substantial percentages of both false-positive and false-negative results (15, 19). Simplified, inexpensive culture devices (5, 14) designed for office use still require at least overnight incubation.

The lysate prepared from the amebocytes of *Limulus polyphemus*, the horseshoe crab, undergoes a gelation in the presence of very minute amounts of endotoxin from gram-negative bacteria (12). The *Limulus* assay is a promising method for rapid detection of endotoxin in patients suspected of having gram-negative septicemia (13). Presently, this method is the most sensitive test known for the detection of endotoxin (4, 18) and can be applied easily to the sampling of fluids other than blood (3). Both solubilized endotoxin and endotoxin associated with the intact cell walls of viable gram-negative bacteria may be detected by this assay (J. H. Jorgensen and R. F. Smith, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 92, 1972).

The purpose of this investigation was to

evaluate the *Limulus* endotoxin assay as a possible method for the rapid detection of significant gram-negative bacteriuria.

MATERIALS AND METHODS

Preparation of Limulus lysate. Horseshoe crabs were purchased from the Marine Biological Laboratory, Woods Hole, Massachusetts. Amebocyte lysate was prepared according to the methods of Reinhold and Fine (17) except that the amebocytes were sedimented at 50 × *g* for 10 min, the cells were washed only twice with pyrogen-free sodium chloride (Travenol Laboratories, Morton Grove, Illinois), and the amebocytes were lysed by the addition of pyrogen-free distilled water (Travenol) in a 1:3 ratio of packed cells to water. All lysate batches used in this study could detect as little as 1 ng of *Escherichia coli* 055:B5 endotoxin (Boivan extract, Difco Laboratories, Detroit, Michigan) per ml.

Performance of Limulus assay. Urine samples were diluted in 10-fold increments (1:10; 1:100; and 1:1,000) in sterile pyrogen-free saline (Travenol). Samples of 0.1 ml of each dilution and of undiluted urine were then added to 0.1-ml volumes of *Limulus* lysate in disposable glass test tubes (10 by 75 mm) (Corning Glass Works, Corning, New York) previously rendered sterile and pyrogen-free. A negative control was performed by including one tube containing 0.1 ml of *Limulus* lysate and 0.1 ml of the saline diluent. The reaction mixtures were then incubated for 60 min at 37 C. After incubation, the presence of a solid gel or a marked increase in viscosity and turbidity represented a positive test for endotoxin (Fig. 1). Weak positive (w+) *Limulus* assay reactions from undiluted urine were those showing only slight increases in viscosity and/or turbidity rather than a firm gel. Weak positive reactions were recorded as such and tallied separately from positive and negative reactions. The highest dilution of urine giving a positive reaction was noted as a means of quantitating the number of bacteria present.

Bacteria utilized. Strains of *Escherichia coli* and *Klebsiella pneumoniae* isolated from urinary tract infections were utilized to generate standard curves of *Limulus* lysate reactivity compared with viable bacterial counts. These organisms were grown in brain heart infusion broth (BBL, Cockeysville, Md.) for 6 h at 37 C in a shaking water bath. The cells were collected by centrifugation at 17,300 × *g* for 15 min, washed once with sterile, pyrogen-free saline, and again sedimented. The cells were then suspended in saline to a density of approximately 10^7 organisms per ml. A 1:10 dilution of the cell suspension was then made by using filter-sterilized (0.22-μm pore size, Millipore Corp.) normal human urine. The resulting cell suspension contained approximately 10^6 cells per ml. Serial 10-fold dilutions were made in urine to include estimated cell densities from 10^6/ml to 10 cells per ml. Each of the six cell suspensions were cultured to obtain exact cell densities for each dilution.

The same bacterial strains were utilized to test the ability of the *Limulus* assay to detect antibiotic-killed cells. The organisms were grown, and the cell densities were adjusted in exactly the same manner as described above. However, 100 μg of gentamicin (Garamycin, Schering Corp., Bloomfield, New Jersey) per ml was added to a suspension of approximately 10^6 cells per ml urine. This cell suspension was then allowed to incubate in the presence of the antibiotic for 2 h at 37 C. Quantitative plate counts were performed on the urine before and after exposure to the antibiotic.

Culture methods for patient urine specimens. Patient urine specimens were processed either within 1 h of their collection or were immediately refrigerated to insure the accuracy of quantitation of bacteria in the sample. Urine specimens were cultured by using the standard-volume calibrated bacteriological loop method (6). Samples of urine from each loop (0.01 and 0.001 ml) were applied to one plate each of 5% human blood in Columbia agar base (BBL) and MacConkey agar (BBL) by using the spread-plate technique of inoculation. The four petri plates were then incubated at 37 C for 18 to 24 h and examined for growth.

Patient group studied. The 64 patients involved in this study were children under the age of 14 years who were either admitted to Shriners Burns Institute with acute thermal injuries, or were patients of the Pediatrics Department of the University of Texas Medical Branch. These included 44 burn patients, 5 spina bifida patients, and 15 general pediatric patients, some of whom were suspected of having acute pyelonephritis.

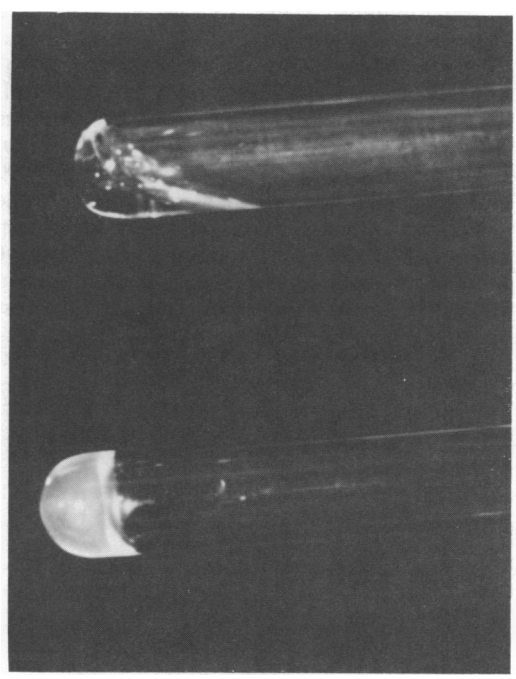

FIG. 1. *Close-up view of a negative Limulus test (top) and a strongly positive test (bottom).*

RESULTS

Measurement of endotoxin associated with viable cells of *E. coli* and *K. pneumoniae* in urine demonstrated that a standard curve of *Limulus* assay reactivity compared with viable cell numbers can be constructed. Based on 10-fold dilutions, a linear relationship between the *Limulus* test and quantitative urine cultures appears to exist over a range of 1,000 to 1,000,000 bacteria per ml (Fig. 2). A positive *Limulus* assay at a dilution of 1:100 or 1:1,000 indicated the presence of greater than 100,000 bacteria per ml. A positive reaction from undiluted urine or at a dilution of only 1:10 indicated less than 100,000 cells per milliliter.

After the 2-h incubation of *E. coli* and *K. pneumoniae* cells in urine containing 100 μg of gentamicin per ml, a positive *Limulus* assay was still obtained at a dilution of 1:1,000 from approximately 10^6 cells/ml (Table 1). Therefore, the nonviability of gram-negative bacterial cells did not affect the ability of the *Limulus* assay to detect and quantitate the number of cells present.

Table 2 shows the results of *Limulus* assays and quantitative cultures on 209 urine samples from 64 different patients. Twenty-five specimens had greater than 100,000 organisms/ml of urine. These included 12 isolates of *E. coli*, 5 of *K. pneumoniae*, 2 *Proteus* species, 1 each of *Pseudomonas*, *Staphylococcus aureus*, and *Candida albicans*, and three specimens containing mixed gram-negative flora totaling 10^5/ml. These data support the experimental findings that a positive endotoxin assay at a dilution of 1:100 or 1:1,000 indicates greater than 100,000 gram-negative bacteria per ml. However, the specificity of the *Limulus* assay for endotoxin did not allow detection of yeast or gram-positive bacteria, even at concentrations of greater than 10^5 cells per ml (Table 2). The *Limulus* test correctly detected 92% of all samples containing greater 100,000 organisms per ml and 100% of the urine specimens containing greater than 10^5 gram-negative bacteria per ml (Table 3). Furthermore, the *Limulus* test accurately classified 99% of the urine samples on the basis of whether they contained greater than or less than 100,000 organisms per ml.

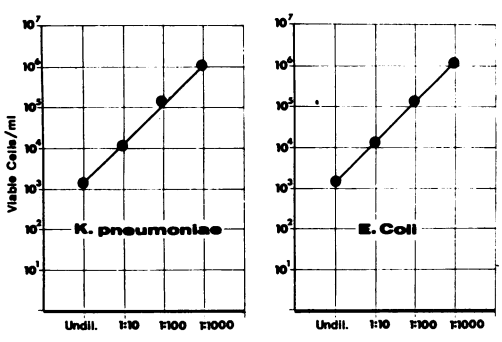

FIG. 2. *Standard curves of Limulus assay results on dilutions of urine containing gram-negative bacilli.*

DISCUSSION

The *Limulus* in vitro endotoxin assay has been shown to be a promising tool for the detection of endotoxin in blood and in parenteral fluids. The results outlined in this investigation indicate that it can also be applied to the assay of urine for the detection of endotoxin associated with gram-negative bacteria. The results of this test can be obtained within 90 min of collection of a urine specimen. Furthermore, the performance of this test does not require the degree of subjective judgement for accurate quantitation of bacteria that is required by methods involving microscopy of urine sediment. The cost of performing the *Limulus* test on urine would be comparable to or less than the cost of a quantitative urine culture in most laboratories. On this basis, the test might prove to be useful for screening patients for asymptomatic bacteriuria.

Since the *Limulus* assay is not affected by the presence of antibiotics and can detect the presence of nonviable gram-negative bacterial cells in urine, it could be used to monitor the effectiveness of treatment of urinary tract infections. The *Limulus* assay has the added advantage of being able to detect the presence of anaerobic gram-negative bacteria (Sonnenwirth et al., Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 94, 1972) that might be overlooked by routine urine culture methods.

One deficiency of the *Limulus* test is that gram-positive bacteria and yeast are undetectable. However, only a small percentage of all urinary tract infections are caused by organisms other than gram-negative bacteria (1, 10). Another disadvantage is that the test provides no information regarding the identity of the organisms, or the presence of mixed bacterial infections. Furthermore, no information regarding the antibiotic susceptibility profile of the organism is derived from a positive *Limulus* test. The *Limulus* assay is therefore proposed not as a replacement for the quantitative urine culture, but rather as a simple, rapid, presumptive method of detecting gram-negative bacteriuria in those patients where an immediate diagnosis is desired.

TABLE 1. *The ability of the Limulus assay to detect antibiotic-killed cells*

Organism	Colony count before gentamicin	Colony count after gentamicin[a]	*Limulus* reaction			
			Undiluted	1:10	1:100	1:1,000
E. coli	1.42×10^6	4	+	+	+	+
K. pneumoniae	1.20×10^6	0	+	+	+	+

[a] Gentamicin, 100 μg/ml. for 2 h at 37 C.

TABLE 2. *Results of Limulus assays and quantitative urine cultures on 209 urine specimens*[a]

Limulus results	Culture results									
	Negative or <100	$10^2 - 10^3$		$10^3 - 10^4$		$10^4 - 10^5$		$10^5 - 10^6$		$> 10^6$	
		GPB	GNB	GPB	GNB	GPB	GNB	GPB	GNB	GPB	GNB
Positive @ Undiluted	6	0	7	1	7	1	3	0	0	0	0
Positive @ 1:10	3	0	0	0	8	0	6	0	0	0	0
Positive @ 1:100	0	0	0	0	0	0	0	0	3	0	0
Positive @ 1:1,000	0	0	0	0	0	0	0	0	5	0	15
w+ @ Undiluted	20	1	7	1[b]	7	1	3	1[b]	0	0	0
Neg. @ Undiluted	71	3[b]	11	5	8	3	0	1	0	0	0

[a] GPB, Gram-positive bacteria; GNB, gram-negative bacteria; w+, weak positive reaction (see text).
[b] Includes one isolate of *Caandida albicans*.

TABLE 3. *Correlation of Limulus assays with quantitative urine cultures*

Results	Agreement (%)
Samples with $>10^5$ organisms/ml having a positive *Limulus* assay (%)	92% (23/25)
Samples with $>10^5$ gram-negative bacteria/ml having a positive *Limulus* assay	100% (23/23)
Overall ability of *Limulus* assay to correctly classify urine specimens as $<10^5$/ml or $>10^5$/ml	99% (207/209)

ACKNOWLEDGMENTS

This study was supported by funds from the Shriners of North America and the James W. McLaughlin Foundation for the Study of Infection and Immunity.

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