Chromogenic *Limulus* Amoebocyte Lysate Assay for Rapid Detection of Gram-Negative Bacteriuria

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A chromogenic *Limulus* amoebocyte lysate assay was evaluated as a rapid screening test for the detection of clinically significant gram-negative bacteriuria. The development of a distinctive yellow color after the addition of chromogenic substrate to the *Limulus* amoebocyte lysate-urine reaction mixture was used to measure $\geq 10^5$ gram-negative bacteria per ml. A total of 324 urine specimens were assayed, with 68 gram-negative urinary tract infections identified as defined by quantitative urine colony counts of $\geq 10^5$ bacteria per ml. Of these, 68 and 67 of 68 were detected by the chromogenic *Limulus* amoebocyte lysate assay at urine dilutions of 1:10 and 1:20, respectively. Nine false-positive chromogenic *Limulus* amoebocyte lysate assay results were observed at both urine dilutions and in the same specimens. At a urine dilution of 1:10, sensitivity and specificity were 100 and 96.6%, respectively, with predictive values of 100% for a negative test and 88.3% for a positive test. At a urine dilution of 1:20, sensitivity and specificity were 98.6 and 96.6%, respectively; predictive values were 99.6% for a negative test and 88.3% for a positive test. These data suggest that chromogenic *Limulus* amoebocyte lysate assay of urine has potential usefulness as a rapid, reliable, and easily performed and interpreted screening test for the diagnosis of clinically significant gram-negative bacteriuria.

Aqueous lysates prepared from the circulating cells (amoebocytes) of the horseshoe crab (*Limulus polyphemus*) gel when exposed to minute amounts of bacterial endotoxin (11). This reaction is the basis of the *Limulus* amoebocyte lysate (LAL) test, which has been shown to have potential usefulness for rapid diagnosis of gram-negative infections in various body fluids, including cerebrospinal fluid (8, 16), urine (17), and urethral and cervical exudates for the presumptive diagnosis of gonorrhea (15, 20, 24, 25).

The biochemical basis of the LAL test is enzymatic (31), with endotoxin, in the presence of calcium, initiating a series of cascading events which result in the activation of a proclotting enzyme present in the lysate (28). The activated clotting enzyme converts soluble proteins (coagulogen) to an insoluble complex (coagulin) (27, 28) by cleaving the *L. polyphemus* coagulogen at the Arg-Lys and Arg-Gly linkage (18, 19, 28, 29), resulting in the release of peptide C and formation of the characteristic gel. The proteolytic activity of the activated clotting enzyme has been shown to be active against a number of synthetic chromogenic substrates (7), hydrolyzing the terminal Gly-Arg-*p*-nitroaniline in the following reaction: substrate + enzyme + H₂O → peptide + *p*-nitroaniline. *p*-Nitroaniline is distinctively yellow, and the rate and amount, and thus the color depth, at which it is liberated is a function of the endotoxin concentration in the test sample. Chromogenic substrates have simplified the use of the LAL assay for the detection and quantitation of endotoxin and may further expand the diagnostic usefulness of the assay, having been shown feasible for the detection of gram-negative bacteremia or endotoxemia or both (5, 30), meningitis (M. A. Saubolle, T. Barone, A. Blattman, and A. Ramos, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, C120, p. 256), and gonococcal urethritis in men (21).

Nachum and Shanbrom (17) have described a LAL gelation assay for the detection of gram-negative bacteriuria. The assay is based on the rapid gelation of LAL by the

relatively large endotoxin concentrations present in undiluted urine infected with gram-negative bacteria. Gel formation within 10 to 15 min was shown to be a reliable indicator of $\geq 10^5$ gram-negative bacteria per ml of urine. The rate at which *p*-nitroaniline is released when synthetic substrates are used is directly proportional to the endotoxin concentration (7). Given the potentially large amounts of endotoxin present in the urine of patients with gram-negative urinary tract infections, a study was undertaken to determine whether the chromogenic LAL (CLAL) assay could be used as a visual screening test for the detection of gram-negative bacteriuria. In the present communication, we present data showing that the CLAL urine assay has potential usefulness as a rapid and reliable screening test for clinically significant gram-negative bacteriuria, as defined by the presence of $\geq 10^5$ bacteria per ml of urine (9).

MATERIALS AND METHODS

Glassware. All glassware used in this study was rendered free of endotoxin by heating in a dry-air oven at 200°C for 4 h.

Patient population. Urine specimens were obtained from both inpatients and outpatients complaining of one or more of the following: dysuria, frequency or urgency of urination, and lower abdominal or flank pain. Urine specimens routinely submitted for hospital admission were also included in the study.

Urine collection and culture. Clean-voided urine specimens were collected by patients in sterile midstream collector kits (Concord Laboratories, Keene, N.H.). These kits contained instructions for use and cleansing towelettes impregnated with benzalkonium chloride. Quantitative urine culture was performed with a calibrated (0.001-ml) loop method (1). Urine specimens for culture were inoculated onto one plate each of MacConkey agar and 5% sheep blood agar (Scott Laboratories, Inc., Carson, Calif.) within 30 min after their receipt in the laboratory. The plates were incubated overnight at 35°C. If no growth was observed after the initial

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TABLE 1. Results of CLAL assay

No. of GNB (CFU/ml) ^a	No. of CLAL-positive specimens/total no. tested at dilution of:	
	1:10	1:20
<10 ³	8/221	8/221
≥10 ³ –<10 ⁴	0/17	0/17
≥10 ⁴ –<10 ⁵	1/18	1/18
≥10 ⁵	68/68	67/68

^a GNB, Gram-negative bacilli.

incubation period, the plates were reincubated for an additional 24 h. Gram-negative isolates (*Enterobacteriaceae*) were identified with Micro-ID panels (General Diagnostics, Morris Plains, N.J.), and nonfermenters and gram-positive microorganisms were identified by standard methods (10). Clinically significant bacteriuria was defined by blood agar colony counts of ≥10⁵ bacteria per ml of urine (9).

CLAL assay. The CLAL assay system used for this study was obtained from Whittaker M. A. Bioproducts, Walkersville, Md., in kit form (QCL-1000). LAL, chromogenic substrate (S-2423), and reference endotoxin were reconstituted according to the instructions of the manufacturer.

CLAL assay of urine. Upon receipt, 1 to 2 ml of the patient urine specimens was poured into sterile polystyrene capped test tubes (12 by 75 mm; Falcon Plastics, Oxnard, Calif.) and held at 4 to 6°C until assay (2 to 4 h). Before assay, urine specimen dilutions of 1:10 and 1:20 were prepared in pyrogen-free water (Travenol Laboratories, Inc., Deerfield, Ill.). CLAL assay of the diluted urine was performed as follows: equal volumes (0.05 ml) of the diluted sample and LAL were pipetted into sterile, endotoxin-free disposable borosilicate glass test tubes (10 by 75 mm) and incubated for 5 min at 37°C in a standing water bath, at which time 0.1 ml of chromogenic substrate, prewarmed to 37°C, was added to the reaction mixture. The tubes were immediately removed from the water bath, hand-shaken for 5 s, and observed for not more than 30 s for development of a strong yellow color (positive result). Lack of color development or the formation of a slight yellow tinge was scored as a negative result. Both positive and negative controls were run with each batch of tests, the latter consisting of pyrogen-free water and the former a known culture-positive urine sample.

Endotoxin concentrations in urine were determined by CLAL assay. A standard endotoxin curve (25 to 100 pg/ml) was obtained with *Escherichia coli* reference endotoxin as supplied in the CLAL kit. Quantitative endotoxin CLAL assay determinations were done by mixing equal quantities (0.05 ml) of the endotoxin reference standards or diluted urine and LAL in endotoxin-free test tubes (10 by 75 mm) and incubation for 10 min at 37°C, at which time 0.1 ml of chromogenic substrate was added and the tubes were gently shaken and reincubated for an additional 3 min at 37°C. The reaction was stopped by the addition of 0.3 ml of a 50% acetic acid solution, the tubes were vortexed, and absorbance was read at 405 nm on a Bausch & Lomb Spectronic 710 spectrophotometer. A negative control consisting of pyrogen-free water was run with each batch of tests, and the optical density of the blank was subtracted from the absorbancies of the test assays. The spectrophotometer was blanked against distilled water.

The minimum concentration of endotoxin per milliliter in culture-positive urine specimens needed to yield a positive CLAL test was determined by twofold dilution in pyrogen-free water, starting at 1:20, until a negative result was

achieved. The last dilution to give a positive result was used to calculate the minimum concentration of endotoxin per milliliter of urine needed to yield a positive result within the constraints of the assay, as described above.

Chloroform extraction. CLAL false-positive urine specimens were chloroform extracted (12) by adding 0.25 ml of chloroform to 0.75 ml of undiluted urine and mixing for 3 h at room temperature, followed by centrifugation at 1,100 × *g* for 10 min. The middle layer was removed, diluted 1:10 in pyrogen-free water, and tested for endotoxin by CLAL assay.

RESULTS

Preliminary experiments were performed to determine optimal CLAL assay conditions and the minimum concentration of endotoxin per milliliter of urine needed to yield positive CLAL assays, with five known culture-positive (≥10⁵ gram-negative CFU/ml) and two culture-negative (<10³ CFU/ml) urine specimens from patients. Data obtained from these experiments established that a 5-min LAL-sample incubation at 37°C gave optimal discrimination between culture-positive and culture-negative urine specimens at sample dilutions of 1:10 and 1:20. Additionally, at these urine dilutions, the natural color of urine was sufficiently quenched so as not to interfere with the visual reading of the assay. Minimum endotoxin concentrations required for a positive CLAL assay, within the constraints of the assay (5 min at 37°C), were found to range from 2.0 to 4.1 ng/ml of urine.

Color development in urine specimens containing ≥10⁵ gram-negative CFU/ml was rapid, with a strong, distinctive yellow color evident within 30 s after addition of the chromogenic substrate. Lack of color development or the evolution of a slight yellow tinge within 30 s was scored as a negative result. Increasing the incubation time to 3 min after the addition of the chromogenic substrate resulted in strong color development in approximately 30% of patient urine specimens subsequently assayed; however, no correlation with colony counts of ≥10⁵ gram-negative bacteria per ml of urine was noted. Thus, visual reading of the assay was recorded 30 s after addition of the chromogenic substrate.

Of the 324 urine specimens cultured and assayed for CLAL activity, 82 were classified as bacteriuric, with 68 (81.6%) of gram-negative etiology, including 26 isolations of *E. coli*, 8 each of *Klebsiella pneumoniae* and *Proteus mirabilis*, 2 each of *Citrobacter diversus* and *Pseudomonas aeruginosa*, and one each of *Morganella morganii*, *Proteus vulgaris*, *Hafnia alvei*, *Enterobacter cloacae*, *Serratia marcescens*, and *Acinetobacter anitratus*. Polymicrobial gram-negative infections were observed in 16 urine specimens.

Results of CLAL assay of all urine specimens are shown in Table 1. At a urine dilution of 1:10, positive CLAL assay results were obtained for all urine specimens containing >10⁵ gram-negative CFU/ml; however, one false-negative reaction was observed at a urine dilution of 1:20. Nine false-positive reactions were recorded at both dilutions and for the same specimens, with eight of these from urine specimens with quantitative gram-negative colony counts of <10³/ml, and one observed at a count of 12,000 gram-negative CFU/ml. Eight of nine of the CLAL false-positive urine specimens contained gram-positive organisms ranging in counts from >10⁴ to >10⁵ CFU/ml (Table 2), with two of these containing probable pathogens, *Staphylococcus aureus* and group D streptococci (enterococci). The remaining

six specimens were considered contaminated, with either single or mixed isolations of viridans group streptococci, diphtheroids, or *Staphylococcus epidermidis*. The one false-positive CLAL assay result for which no organisms were isolated ($<10^3$ CFU/ml) was obtained from a male patient; the other eight were obtained from female patients.

Chloroform extracts were prepared from the nine urine specimens for which false-positive results were obtained. All were successfully retested at a dilution of 1:10 by CLAL assay, indicating that the positive CLAL assay specimens were caused by endotoxin present in the urine specimen. To rule out the possibility that endotoxin contamination of the midstream collector jars may have contributed to the endotoxin load of these urine specimens, 20 ml of pyrogen-free water was added to each of five jars and shaken vigorously, and portions were removed and assayed for endotoxin by quantitative CLAL assay. No measurable endotoxin was detected in any of the samples.

Endotoxin concentrations were determined for urine specimens containing $>10^5$ gram-negative CFU/ml (20 specimens), urine specimens for which false-positive results were obtained (9 specimens), and for urine specimens with $<10^3$ gram-negative CFU/ml (10 specimens). Urine specimens with $>10^5$ gram-negative CFU/ml were found to contain high concentrations of endotoxin, ranging from 200 to 2,150 ng/ml. Endotoxin concentrations in CLAL false-positive urine specimens ranged from 40 to 175 ng/ml. In contrast, true-negative urine specimens ($<10^3$ CFU/ml, CLAL negative) contained endotoxin concentrations ranging from a high of 20 to a low of 3.5 ng/ml.

Predictive values (22) for detection of clinically significant gram-negative bacteriuria by CLAL assay are given in Table 3.

DISCUSSION

The rate of LAL gelation increases with increasing concentrations of endotoxin (3, 11). This relationship provided the basis for a previously published study (17) in which rapid LAL gelation (10 to 15 min) was used for detection of urine specimens containing 10^5 gram-negative CFU/ml. In the CLAL test, hydrolysis of the colored complex *p*-nitroaniline from a synthetic substrate is similarly increased by increasing the concentration of endotoxin (7); it was thus postulated that rapid color development might be used as a reliable indicator for screening of urine specimens containing clinically significant numbers of gram-negative bacteria. Preliminary

TABLE 3. Predictive values for detection of gram-negative bacteriuria by CLAL assay^a

Dilution	% Sensitivity	% Specificity	% Predictive value	
			Positive	Negative
1:10	100	96.6	88.3	100
1:20	98.6	96.6	88.3	99.6

^a Predictive values were calculated as follows: sensitivity, true-positive/(true-positive + false-negatives) $\times$ 100; specificity, true-negative/(true-negative + false-positives) $\times$ 100; predictive positive, true-positive/(true-positive + false-positives) $\times$ 100; predictive negative, true-negative/(true-negative + false-negatives) $\times$ 100.

experiments established that 5 min of incubation of urine-LAL at 37°C was sufficient to differentiate urine specimens on the basis of $<10^5$ and $>10^5$ gram-negative CFU/ml. For assay, urine dilutions of not less than 1:10 were necessary for the following reasons: (i) the natural color of most urine specimens would either mask reading of a positive CLAL assay or, in the absence of bacteriuria, might be interpreted as a positive test; and (ii) low levels of endotoxin shown to be present in urine specimens containing $<10^3$ CFU/ml might result in false-positive CLAL results. The latter point was confirmed by quantitative data obtained from culture-negative urine specimens, with endotoxin concentrations ranging from 3.5 to 20 ng/ml, amounts shown sufficient to yield positive CLAL assay results in undiluted specimens. Furthermore, endotoxin concentrations of 2.0 to 4.1 ng/ml in urine were shown to result in positive CLAL assay results within the constraints of the assay procedure, concentrations well within the range shown for nonbacteriuric urines. Thus, dilution of the urine was essential to avoid large numbers of false-positive results.

Nine false-positive CLAL assay results were observed out of a total of 324 urine specimens tested. Chloroform extracts of these specimens were still LAL reactive, indicating that the false-positive CLAL assay results were endotoxin mediated and not the result of nonspecific conversion of the lysate proclotting enzyme to its active form. Of the nine false-positive results recorded, eight were observed for urine specimens obtained from female patients. Two of these specimens contained probable gram-positive pathogens; however, the remaining six were obviously contaminated, as evidenced by the mixed gram-positive flora cultured, indicating improper cleansing before voiding (1). It is possible that the endotoxin detected in these urine specimens was extraneous, i.e., originating from the midstream collector jars, or was the result of in vivo contamination of the specimen. The midstream collector jars were ruled out experimentally as a potential source of endotoxin; thus, it appears that the false-positive results were a result of in vivo contamination of the urine specimen during collection, i.e., either the external (vulva or perineum) or internal (vagina, cervix) genitalia or both. Both sites are known to harbor an indigenous microflora of gram-positive and -negative bacteria (6, 14). Additionally, the normal female urethra is frequently colonized by gram-negative organisms (4). These anatomical sites commonly contribute to the bacterial load of urine specimens obtained for culture, especially when adequate cleansing procedures are relaxed (1). Because urine culture was performed at a dilution of 1:1,000 only, gram-negative counts of less than 1,000/ml could not be ascertained; however, low numbers of gram-negative bacteria or free endotoxin or both might easily have contributed to the endotoxin load (3.5 to 20 ng/ml) shown to be present in

TABLE 2. Organisms isolated from urine specimens yielding false-positive CLAL assay results

Specimen no.	Sex of patient	Organism(s) isolated	No. of CFU/ml
1	F	<i>S. aureus</i>	$>10^5$
2	F	Diphtheroids	$>10^5$
3	F	Enterococci	$>10^5$
4	F	Diphtheroids	$>10^4$ – $<10^5$
		Gram-negative bacilli	1.2×10^4
5	F	Diphtheroids	$>10^4$ – 10^5
6	F	Diphtheroids	$>10^4$ – 10^5
7	F	Diphtheroids, viridans streptococci, <i>S. epidermidis</i>	$>10^5$
8	F	<i>S. epidermidis</i> diphtheroids	$>10^5$
9	M	None	$<10^3$

true-negative urine specimens ($<10^3$ CFU/ml, CLAL negative).

The only culture-negative urine specimen ($<10^3$ CFU/ml) that yielded a false-positive CLAL assay result was obtained from a male patient. Previously (17), two culture-negative, false-positive LAL urine assay results were described, both for specimens obtained from male patients who had culture-proven gonococcal urethritis. Barring gross contamination of the specimen, it is possible, albeit speculative, that the patient in the present study may have been suffering from either gonococcal or *Chlamydia trachomatis* urethritis, the latter shown to contain endotoxin and to cause gelation of LAL (13). The large amount of endotoxin (175 ng/ml) found in this patient's urine, as compared with a mean of 60 ng/ml $\pm$ 19 present in urine specimens obtained from females and yielding false-positive CLAL assay results, might be viewed as further evidence for urethritis of either gonococcal or chlamydial etiology.

Endotoxin concentrations shown to be present in bacteriuric urine specimens suggest that a considerable percentage of the endotoxin load exists as free endotoxin, i.e., is not cell bound. Endotoxin concentrations per cell of *E. coli* range from 32.5 fg/ml during the logarithmic phase of growth to a low of 10.0 fg/ml in the stationary phase (26). It thus appears unlikely that gram-negative urine colony counts of 1×10^5 to 2×10^5 or greater could account for the endotoxin concentrations reported in urine. These high endotoxin concentrations suggest that dilutions greater than 1:20 would still yield positive assays in bacteriuric urines, thus possibly obviating some of the false-positive CLAL assay results reported in this study.

As presently constituted, CLAL assay of urine has advantages over the use of gel endpoints, providing the user with a more rapid turnaround time, an important aspect for any screening test, and an easily read endpoint. Additionally, problems associated with LAL gel endpoint assays, such as inadvertent dissolution of the gel during either incubation or reading, are obviated by chromogenic assay (17, 23).

The results of this investigation have shown that CLAL assay of urine offers a sensitive and reliable screening test for the rapid diagnosis of clinically significant gram-negative bacteriuria for the following reasons: (i) high predictive values are reported for both a positive and a negative assay; (ii) the great majority of bacteriurias are of gram-negative etiology (2); and (iii) the assay is easily performed and interpreted. Thus, CLAL assay could prove useful for routine urine screening in outpatient clinics and physician office practices.

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