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

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The *Limulus* amoebocyte lysate (LAL) test was evaluated for rapid detection of gram-negative bacteriuria in an adult patient population. Time to gelation of a standard LAL preparation was used as a measure of significant (>10⁵ bacteria per ml) gram-negative bacteriuria, and the results of 19% LAL assays were compared with quantitative urine cultures. Initially, 33 of 36 urine specimens containing >10⁵ gram-negative bacteria per ml were detected by LAL assay. The three false-negative LAL tests were the result of urine pH levels below the pH minimum for LAL gelation; neutralization of these urine specimens resulted in positive LAL assays and 100% correlation with culture results. All 36 bacteriuric urine specimens were LAL positive within 15 min, with the majority of assays (86.1%) being positive after only 10 min of incubation at 37°C. These data compared favorably with gelation times of 15 min when 1×10⁵ to 2×10⁵ gram-negative bacteria per ml were added to sterile urine. Two urine samples obtained from male patients with culture-proven gonococcal urethritis yielded positive LAL assays. The LAL assay was shown to correctly differentiate 96.2% of urine specimens as containing <10⁵ or >10⁵ gram-negative bacteria per ml. The results of this study have shown that the LAL test can be used as a rapid, simple, and reliable screening procedure for the diagnosis of clinically significant gram-negative bacteriuria.

Aqueous extracts of *Limulus polyphemus* (horseshoe crab) amoebocytes, known as *Limulus* amoebocyte lysate (LAL), gel in the presence of minute amounts of bacterial endotoxin (9), a property which has found wide application as a potentially useful tool for detection of gram-negative infection in various body fluids and for pyrogen testing by the pharmaceutical industry (15). Initially, the diagnostic usefulness of the LAL assay was shown for detection of gram-negative sepsis (10), an application which has been the subject of numerous conflicting reports. The most successful diagnostic use of the LAL test has been for detection of gram-negative bacterial meningitis, first reported by Nachum et al. (11) and later confirmed by other investigators (5, 14). Rapid detection of clinically significant gram-negative bacteriuria has also been described (3, 4), and a reliable and simple method for detection of gonococcal urethritis by LAL assay has been reported (12, 13, 16).

Kass (16) has defined clinically significant bacteriuria as urine containing more than 10⁵ bacteria per ml. Processing of urine specimens by conventional bacteriological methods requires a minimum of 18 to 24 h before quantitative results are available. This lengthy turnaround time and the lack of facilities for urine culture in the offices of many physicians and outpatient clinics argue for the availability of a rapid, simple, and reliable screening test that would provide the clinician with a useful alternative to more conventional modes for detection of clinically significant bacteriuria. Jorgensen and co-workers (3, 4) have described one such method in which patient urine is diluted, reacted with LAL, and incubated for 1 to 2 h. This assay showed a high degree of correlation between a positive LAL test and clinically significant bacteriuria.

The present investigation was undertaken to determine whether time to gelation of a standard LAL preparation could be used as a reliable method for detection of gram-negative bacteriuria.

**MATERIALS AND METHODS**

**Patient group.** Urine specimens were randomly obtained from outpatient clinic patients at Martin Luther King, Jr., General Hospital, Los Angeles, Calif.

**Collection and culture of patient urine.** Urine specimens were collected by patients in sterile midstream collector kits (Concord Laboratories, Keene, N.H.) containing instructions for use and benzalkonium chloride cleansing towelettes. Patient urine specimens were cultured by a calibrated loop procedure.
within 1 h after receipt in the laboratory. A 0.001-ml amount of urine was inoculated to one plate each of MacConkey agar and 5% sheep blood agar (CalLabs, North Hollywood, Calif.). Cultures were examined after overnight incubation at 35°C and again at 48 h if no growth was observed after the initial incubation period. Identification of bacterial isolates was done by standard methods (8). As defined by Kass (6), colony counts of >10^5 bacteria per ml were considered to indicate clinically significant bacteriuria.

**Urine pH.** Urine pH was not routinely determined. Determinations of pH were done with Multistix reagent strips (Miles Laboratories, Inc., Elkhart, Ind.) only on bacteriuric specimens found to be LAL negative (3 of 36). Bacteriuric specimens shown to be below a pH level of 6.0 were neutralized with endotoxin-free 0.1 N NaOH (Sigma Chemical Co., St. Louis, Mo.).

**LAL assay.** LAL used in this study was provided by Mallinckrodt, Inc., St. Louis, Mo. (lot 812-XODY). LAL was supplied as a lyophilized preparation in individual test vials. Assays were run by the addition of 0.25 ml of test solution to the test vials, as recommended by the manufacturer. The sensitivity of LAL was determined to be 0.5 ng/ml with *Escherichia coli* EC-2 endotoxin.

**LAL assay.** Upon receipt in the laboratory, patient urine specimens were immediately aliquoted into sterile, plastic, capped test tubes (Falcon Plastics, Oxnard, Calif.) for LAL assay. All urine samples were kept at 4 to 6°C and were assayed within 4 h after collection. LAL assay was performed by adding 0.25 ml of undiluted urine to the test vial and by incubating the vial in a standing water bath at 37°C for a period not exceeding 60 min. Both positive and negative controls were run with each batch of tests. The positive control consisted of 0.5 ng of EC-2 endotoxin per ml; negative controls were performed with pyrogen-free water (Travenol Laboratories, Inc., Deerfield, Ill.). After incubation, the reaction mixtures were carefully removed from the water bath and inverted 180°. A solid gel adhering to the bottom of the tube after inversion indicated a positive test. A negative test was recorded when the reaction mixture ran down the side of the tube.

**Bacteria.** The following American Type Culture Collection (ATCC) bacterial strains were used in this study: *E. coli* 25922, *Klebsiella pneumoniae* 13883, *Serratia marcescens* 8100, *Enterobacter cloacae* 23355, and *Streptococcus faecalis* 8043. ATCC strains were derived from Bactrol disks (Difco Laboratories, Detroit, Mich.) and rehydrated according to the manufacturer's instructions; they were subsequently subcultured to tryptic soy agar (Difco Laboratories) and checked for purity.

**Time-to-gelation determinations.** Bacterial species used for these experiments were inoculated to 0.5 ml of brain heart infusion broth (Difco Laboratories) and incubated for 5 h at 37°C. After incubation, the cell suspension was diluted in filter-sterilized (Millex, 0.22 µm, Millipore Corp., Bedford, Mass.) normal urine to yield an inoculum of 1 × 10^6 to 2 × 10^10 colony-forming units (CFU) per ml. Further dilutions were performed in sterile urine to yield bacterial counts of 5 × 10^4, 1 × 10^4, and 1 × 10^3 CFU/ml. Viable counts were performed as a check on inoculum size by a pour plate method, with tryptic soy agar as the growth medium. All datum points were done in duplicate, and the counts were averaged.

To determine the time to gelation, 0.25-ml samples of the desired urine-cell suspension were added to the LAL test vials and incubated for various lengths of time. Initially, four replicate tests were run for each test organism, with counts ranging from 10^8 to >10^9 CFU/ml to bracket gelation time. Further tests were then run to more specifically determine gelation time. Each datum point obtained was the result of two separate experiments.

**RESULTS**

Four species of gram-negative bacteria suspended separately in sterile urine at counts ranging from 1 × 10^6 to 2 × 10^9 to 1 × 10^6 to 2 × 10^9 CFU/ml were used to determine the time required for gelation of a standard LAL preparation (Table 1). In addition, a gram-positive species (enterococci) and un inoculated urine were included as controls. At an inoculum size consistent with bacteriuria (>10^6 CFU/ml), gelation time did not exceed 15 min for the gram-negative species tested; however, bacterial counts below 10^6 CFU/ml resulted in considerably longer gelation times. In contrast, neither the gram-positive control nor the un inoculated urine produced a positive LAL test within the 60-min maximum incubation time.

Preliminary experiments were conducted to determine gelation time of undiluted patient urine specimens, and results were compared with data shown in Table 1 and with quantitative urine culture results. Data obtained from these preliminary studies showed that patient urine containing >10^3 gram-negative bacteria per ml caused rapid gelation of the LAL, with the maximum gelation time observed to be 15 min. Accordingly, two tubes of LAL were run for...
each patient urine tested, and tubes were read at 10 and 15 min. Table 2 shows the results of LAL assays and quantitative urine cultures on 190 patient urine specimens. A total of 36 specimens had $>10^5$ gram-negative bacteria per ml, including 25 isolations of *E. coli* (3 of which contained a second gram-negative organism in counts ranging from $10^3$ to $10^4$ CFU/ml), 4 each of *K. pneumoniae* and *Proteus mirabilis*, and 1 each of *Hafnia alvei*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens*. Of these, 33 positive LAL assays were obtained within the 15-min incubation time. Four of six specimens with counts ranging from $5 \times 10^4$ to $<10^5$ gram-negative bacteria per ml were LAL positive. Urine specimens with counts of less than $5 \times 10^4$ gram-negative bacteria per ml were all LAL negative within the 15-min incubation time, as were all specimens containing gram-positive bacteria.

The effect of dilution on gelation time of 10 patient urine specimens containing $>10^5$ gram-negative bacteria per ml, diluted 1:10 and 1:50, was determined. At both dilutions, positive LAL assays were observed within the 15-min incubation period.

The majority of the patient urine specimens with quantitative counts of $>10^5$ gram-negative bacteria per ml yielded positive LAL tests after only 10 min of incubation, with 31 out of 36 specimens (86.1%) positive after 10 min. After 15 min, the remaining five specimens gave positive results. Five aberrant LAL reactions were observed. Two false-positive assays were obtained from specimens with quantitative counts of $<10^5$ gram-negative bacteria per ml. Both of these patients were male, and urethral exudates obtained from these individuals grew out *Neisseria gonorrhoeae*. Three false-negative LAL tests were observed in specimens with counts of $>10^5$ gram-negative bacteria per ml. In all three situations, *E. coli* was isolated. Measurement of urine pH revealed that the pH was below 6.0. Neutralization of the urine to pH 7.0 with endotoxin-free 0.1 N NaOH resulted in positive LAL tests after 10 min incubation.

Data regarding the ability of the LAL test to correctly classify gram-negative bacteriuria are shown in Table 3. The LAL test detected 100% of all specimens containing $>10^5$ gram-negative bacteria per ml (after pH neutralization of the three false-negative assays) and correctly differentiated 96.2% of all specimens containing gram-negative bacteria as containing $>10^5$ or $<10^5$ bacteria per ml.

### DISCUSSION

The LAL test has previously been shown by Jorgensen and co-workers (3, 4) to be a useful diagnostic tool for detection of gram-negative bacteriuria. These investigators reported that a positive LAL test obtained after a 60-min incubation of urine diluted 1:100 or 1:1,000 correlated with quantitative urine cultures of $>10^5$ gram-negative bacteria per ml. Findings described in this investigation further confirm the usefulness of the LAL test for detection of clinically significant bacteriuria and, furthermore, show that it is possible to diagnose gram-negative bacteriuria rapidly by taking advantage of the relationship between gelation rate and endotoxin concentration. Increasing concentrations of endotoxin have been shown to increase the rate at which

<table>
<thead>
<tr>
<th>Test group</th>
<th>Observed</th>
<th>Expected</th>
<th>Agreement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine specimen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$&gt;10^5$ gram-negative bacteria per ml</td>
<td>36+</td>
<td>36+</td>
<td>100</td>
</tr>
<tr>
<td>$&lt;10^5$ to $&lt;10^4$ bacteria per ml</td>
<td>121-</td>
<td>127-</td>
<td>95.3</td>
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<tr>
<td>$&gt;10^4$ gram-positive bacteria per ml</td>
<td>7-</td>
<td>7-</td>
<td>100</td>
</tr>
<tr>
<td>Overall ability of LAL assay to differentiate urine specimens as having $&gt;10^5$ or $&lt;10^5$ gram-negative bacteria per ml</td>
<td>150</td>
<td>156</td>
<td>96.2</td>
</tr>
</tbody>
</table>

* The chi-square test was significant, with $P < 0.01$ for positive LAL tests of urine specimens with counts of $>10^5$ gram-negative bacteria per ml, as compared with negative LAL tests of urine with counts ranging from $<10^5$ to $<10^5$ gram-negative bacteria per ml. +, Positive result; -, negative result.

* Initially, 3 out of 36 bacteriuric specimens were shown to be LAL negative because urine pH was below the pH minimum of the LAL test; neutralization with endotoxin-free NaOH resulted in positive LAL assays within 15 min time frame and a 100% correlation with culture data.

### Table 2. Results of LAL assay on 190 urine specimens

<table>
<thead>
<tr>
<th>Bacterial concn (CFU/ml)</th>
<th>LAL positive/total tested</th>
<th>GBP*</th>
<th>GNB*</th>
</tr>
</thead>
<tbody>
<tr>
<td>$&lt;10^5$</td>
<td>2/100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^5$ to $4 \times 10^4$</td>
<td>0/23</td>
<td>0/14</td>
<td></td>
</tr>
<tr>
<td>$5 \times 10^4$ to $&lt;10^5$</td>
<td>0/4</td>
<td>4/6</td>
<td></td>
</tr>
<tr>
<td>$&gt;10^5$</td>
<td>0/7</td>
<td>33/36</td>
<td></td>
</tr>
</tbody>
</table>

* GBP, Gram-positive bacteria; GNB, gram-negative bacteria.
gelation of LAL takes place (2, 9).

Results obtained in this study have shown that the LAL assay can detect gram-negative bacteriuria in undiluted urine within a 15-min incubation time, with the majority of assays being positive after 10 min of incubation. Three false-negative LAL tests were observed, all of which were shown to be a result of urine being below the pH minimum of the LAL test (17). Neutralization of the urine specimens resulted in positive LAL assays and 100% correlation with culture results. Culture-negative urine (on MacConkey agar and on blood agar) obtained from two male patients with culture-proven gonococcal urethritis yielded positive LAL assays, suggesting either the presence of large numbers of gonococci in the urine, the release of a considerable amount of cell-free endotoxin, or the entry of gonococci into the urine during urination. Four urine samples containing between 5 × 10^4 and <10^5 gram-negative bacteria per ml were LAL positive, a finding not inconsistent with data reported by Jorgensen and Jones (4).

Preliminary data have demonstrated that patient urine specimens containing >10^5 gram-negative bacteria per ml can be diluted and still yield a positive LAL assay within the 15-min incubation time; however, dilution of urine specimens to which >10^5 gram-negative CFU/ml had been added resulted in gelation times beyond the 15-min incubation period. These data suggest that a considerable amount of free endotoxin is liberated into the urine during active infection. Sullivan et al. (17) have shown that cell-free endotoxin accumulates in growth medium during log-phase growth. This observation suggests that dilution of patient urine specimens could be made without loss of the diagnostic value of the 15-min time for a positive LAL assay. Furthermore, the use of an endotoxin-free buffer diluent, to neutralize urine samples not within the pH limits of the LAL test, would obviate the false-negative reactions described in this report.

This investigation has shown that the LAL test is a reliable, simple, and rapid assay for the diagnosis of clinically significant gram-negative bacteriuria. Its potential usefulness as a screening procedure, as described in this report, is evident for the following reasons: (i) microbiological analysis for bacteriuria constitutes a large part of the work load in clinical microbiology laboratories and in outpatient clinics; (ii) a large majority of cases of bacteriuria are of gram-negative etiology; (iii) the assay has a high degree of specificity and sensitivity; (iv) results can be rapidly obtained, a critical factor for an effective screening test; and (v) the assay is easily performed by nursing or laboratory personnel and has an easily read and interpreted endpoint (i.e., gelation versus no gelation), although the amorphous character of the clot and its somewhat fragile nature require that some care be exercised to avoid inadvertent dissolution of the clot when the reaction tube is inverted 180°.

The major deficiency of the LAL test is its inability to detect bacteriuria caused by gram-positive bacteria and yeasts; however, the majority of bacteriuria cases are of gram-negative etiology, with bacteriuria of gram-positive etiology constituting only a small percentage of cases of this clinical disease (1, 7). Also, the presence of polymicrobial infection with gram-negative bacteria or with gram-negative and gram-positive bacteria cannot be ascertained with the LAL assay.

The LAL test is not intended to replace more conventional methodologies but to provide clinicians and laboratories with a useful test for rapid, reliable, and cost-effective urine screening for gram-negative bacteriuria.

LITERATURE CITED


