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Application of a *Limulus* Test Device in Rapid Evaluation of Gonococcal and Nongonococcal Urethritis in Males

RICHARD B. PRIOR[1]* AND VINCENT A. SPAGNA[1, 2]

Division of Infectious Diseases, Department of Medicine, The Ohio State University College of Medicine, Columbus, Ohio 43210,[1] and the Venereal Disease Clinic, City Department of Health, Columbus, Ohio 43215[2]

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A test device incorporating *Limulus* amoebocyte lysate (Mallinckrodt, Inc., St. Louis, Mo.) was developed for the rapid, presumptive diagnosis of gonococcal and nongonococcal disease in males. The device, which was evaluated in 550 men with exudative urethritis, consisted of a specimen collection syringe, a dilution reservoir containing 10 ml of pyrogen-free water, and a *Limulus* amoebocyte lysate single-test vial. After specimen collection, the syringe was affixed to the dilution reservoir for rapid, accurate dilution of the clinical sample. Contamination of the specimen and potential biohazards to the user were prevented. The diluted sample was then transferred (via the collection syringe) to the lysate test vial for assay of endotoxin. Various incubation times at 37°C were also studied in an additional 301 male patients, and time was reduced from the standard 60 to 30 min while still retaining equivalent predictability of culture results ($P > 0.05$). Of the 550 males evaluated with the test device, 366 had positive cultures for *Neisseria gonorrhoeae*, and 184 were negative. A sensitivity of 99.2% and a specificity of 96.7% were obtained with the test device. Overall ability to predict culture results was 98.4%. Gram-stain sensitivity and specificity were 96.4% and 99.5%, respectively, with an overall accuracy of 97.5%. There were no statistical differences between the *Limulus* amoebocyte lysate test and Gram stain in predicting cultures ($P > 0.05$). Thus, use of the *Limulus* amoebocyte lysate test device would enable the private physician to make an accurate, presumptive diagnosis of gonococcal and nongonococcal disease in males with exudative urethritis within 30 min without the need of a microscope and to initiate proper therapy during the patient's initial evaluation.

Gonorrhea remains epidemic, with over 1,000,000 cases reported annually in the United States (5). Of these cases, over 600,000 occur in men, and it is estimated that four to five times this number are not reported but are seen in the private sector by practicing physicians (1, 6, 9). Moreover, nongonococcal urethritis is now estimated to be nearly twice as frequent in men as gonorrhea (20, 21), and there is sufficient overlap between the signs and symptoms of gonococcal and nongonococcal urethritis that differentiation cannot be made accurately on clinical grounds alone (3, 7, 8). Because single-dose therapy is preferred in treating gonococcal urethritis, primarily due to the reluctance of patients to comply with multiple-dose regimens, and because single-dose therapy is not effective in treating nongonococcal urethritis, efforts must be made to diagnose urethritis accurately. Therefore, the need exists for a simple, rapid, and accurate test to differentiate between gonococcal and nongonococcal urethritis, especially in situations in which Gram stain facilities are unavailable, so that appropriate therapy can be given.

We have demonstrated the successful use of the *Limulus* amoebocyte lysate (LAL) assay for the rapid, presumptive diagnosis of gonococcal and nongonococcal urethritis in over 800 men with exudative urethritis (10, 11, 16, 17). Sensitivity and specificity were greater than 99% and 96%, respectively, with an overall ability to predict culture results of over 98%. However, in those studies we used test tubes and pipettes to make the proper dilution of clinical specimens for the LAL assay, and such a procedure required careful attention to laboratory methodology to prevent contamination of the test specimens with exogenous endotoxins. We report here the development and evaluation of a test device in which the clinical specimen was rapidly and accurately diluted in a self-contained system and tested by the LAL assay. The minimum

time needed for incubation of the specimen was also evaluated.

MATERIALS AND METHODS

Study population. A total of 851 men with uncomplicated exudative urethritis seen at the Columbus Health Department Venereal Disease Clinic were evaluated; 301 men were included in the preliminary incubation time study, and the remaining 550 men were evaluated with the LAL test device. These patients had sought treatment because of urethral discharge or dysuria or both and were selected on a random basis. A minimum of 0.015 ml of urethral exudate was needed for acceptance into the study. Urethral exudate was classified clinically as (i) profuse, spontaneous discharge visible without massage; (ii) moderate, discharge not obvious but visible upon compression of the glans; or (iii) scant, discharge obtained only after urethral massage. Patients who had received antibiotics within 10 days of presentation were excluded. Standardized patient interviews included demography, sexual and venereal disease histories, present illness, and an examination of the genitals and inguinal lymph nodes.

LAL incubation time study. To determine whether an incubation time shorter than the standard 60 min could be used, samples from 301 male patients with exudative urethritis were collected and assayed as previously described (17). Each sample was incubated at 37°C in a heating block and tested at 10-min intervals up to 60 min. Results were correlated with Gram stain and culture results.

LAL test device and procedure. The LAL test device consisted of a specimen collection syringe, a sealed dilution reservoir containing 10 ml of pyrogen-free water, and a lysate single-test vial. The volume of diluent was determined from previously published data from quantitative LAL assays performed on clinical specimens (11). A constant representing the minimum amount of endotoxin in urethral exudate needed to predict gonorrhea was determined, and an equation was formulated to relate sample dilution to lysate sensitivity. The equation is as follows: $D = k/MS$ where D is the dilution of clinical sample (± 1 twofold amount), assuming a collection of 0.025 ml exudate; k is the constant endotoxin concentration of 100 ng/ml; and MS is the minimum sensitivity of lysate in nanograms per milliliter (lot EC-2, Bureau of Biologics, U.S. Food and Drug Administration). For example, given a lysate sensitivity of 0.25 ng/ml, the proper dilution needed would be 1:400, which corresponds to a diluent volume of 10 ml. Two lots of lysate, XODY and XOHA, with minimum sensitivities of 0.5 and 0.29 ng of EC-2 per ml, respectively, were used with this dilution and were within the limits specified by the equation. The numbers of patients evaluated with XODY and XOHA were 325 and 225, respectively.

The dilution reservoir contained a frangible membrane which was broken before use. The sample was collected at the urethral meatus by using a syringe and gentle aspiration until the exudate filled the syringe tip from one-half to full (approximately 0.015 to 0.025 ml). The syringe was then affixed to the dilution reservoir, and the sample was expressed into the reservoir (Fig. 1). The intact syringe-reservoir assembly was shaken and inverted, and the contents were mixed by moving the plunger of the syringe in and out a minimum of five times. After adequate mixing, 0.25 ml of the diluted sample was removed and transferred to the LAL vial by using the collection syringe. The LAL vial was gently swirled, incubated undisturbed at 37°C for 30 min, and read. A positive test was defined as the formation of a gel which remained adherent to the bottom of the vial when carefully inverted 180°; the absence of firm gelation was interpreted as a negative test. The LAL tests were performed within 3 h of sample collection and were read without prior knowledge of the microbiological findings.

Clinical laboratory procedures. After collection of exudate for LAL assay, samples were also collected for Gram stain and for culture of *Neisseria gonorrhoeae* on Thayer-Martin medium. The diagnosis of gonococcal urethritis was based on a positive culture for *N. gonorrhoeae*. A detailed description of the methods used and criteria followed for smear interpretation have been previously described (17). Cultures for viruses or chlamydiae were not done.

Statistical analysis. The results of LAL assay, Gram stain, and culture obtained for each patient were entered into a Hewlett-Packard Model 9825A programmable calculator for subsequent determination of correlation. The sensitivity and specificity of the LAL assay and Gram stain were computed assuming that the culture method was 100% accurate. The chi-square

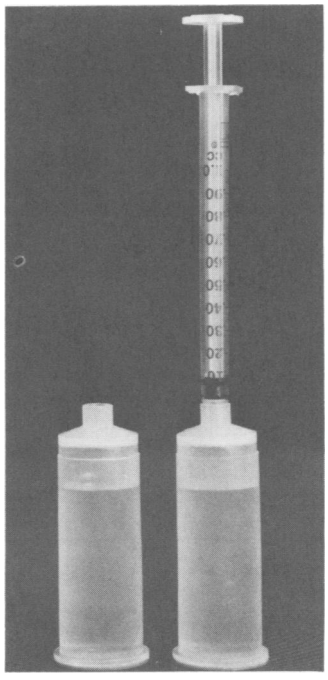

FIG. 1. *Photograph showing sealed dilution reservoir with 10 ml of pyrogen-free water (left) and dilution reservoir with specimen collection syringe attached (right).*

method of analysis was used for the determination of statistical significance.

RESULTS

LAL incubation time study. Selected urethral samples from patients with gonorrhea produced no gelation at 10 min, and only 77% produced a gel at 20 min. Therefore, these two incubation times were not further evaluated. The predictability of culture results at the other four incubation times studied is shown in Table 1.

Of the 301 patients studied, 175 had culture-proven gonococcal urethritis, and 126 had nongonococcal urethritis. At the 30-min incubation time, sensitivity and specificity of the LAL assay were 98.9% (173 of 175) and 97.6% (123 of 126), respectively. Overall accuracy in predicting culture results was 98.3% (296 of 301). Gram-stain sensitivity and specificity were 98.3% (172 of 175) and 99.2% (125 of 126), respectively, with an overall accuracy of 98.7% (297 of 301). There were no statistical differences between the LAL assay and the Gram stain in predicting culture results ($P > 0.05$). The 30-min incubation time was subsequently used for the LAL test device evaluation.

LAL test device evaluation. The results obtained with the LAL test device and two lots of lysate for 550 patients with exudative urethritis are shown in Table 2. Of these 550 patients, 366 had culture-proven gonococcal urethritis, and 184 had nongonococcal urethritis. The sensitivities and specificities were 98.6% (209 of 212) and 96.5% (109 of 113), respectively, for lysate lot XODY and 100% (154 of 154) and 97.2% (69 of 71), respectively, for lysate lot XOHA. Overall accuracy in predicting culture results for lysate lots XODY and XOHA was 97.8% (318 of 325) and 99.1% (223 of 225), respectively. Combined, the LAL assay had a sensitivity of 99.2% (363 of 366), a specificity of 96.7% (178 of 184), and an overall accuracy of 98.4% (541 of 550). There were no statistical differences between lysate lots in ability to predict culture results ($P > 0.05$). Combined, Gram stain had a sensitivity of 96.4% (353 of 366), a specificity of 99.5% (183 of 184), and an overall accuracy of 97.5% (536 of 550). There were no statistical differences between the LAL assay and Gram-stain results ($P > 0.05$).

The clinical descriptions of urethral exudate from 550 patients evaluated with the LAL test device are summarized in Table 3.

TABLE 1. *Effect of various incubation times at 37°C on the predictability of culture results by the LAL assay for 301 male patients with exudative urethritis*

Incubation time (min)	No. of discrepant results[a]	% Predictability of culture
30	5	98.3
40	6	98.0[b]
50	6	98.0[b]
60	8	97.3[b]

[a] Combined false-positive and false-negative LAL assay results as compared to culture results.

[b] Not statistically different from the 30-min results ($P > 0.05$).

DISCUSSION

Accurate differentiation between gonococcal and nongonococcal urethritis cannot be made on clinical grounds alone due to overlap in signs and symptoms (3, 7, 8). In this study, clinical evaluation was a poor index of diagnostic accuracy (Table 3). However, in most cases an immediate and firm diagnosis can be made with the aid of the Gram stain (8). Gram-stained smears performed on urethral exudates from men with symptomatic urethritis and interpreted by experienced personnel are highly sensitive (93 to 99%) and highly specific (98%), with an overall accuracy of approximately 98% (8, 12, 15). Our experience with the Gram stain and trained microbiologists to examine and interpret the smears also demonstrates this high degree of accuracy, with an overall ability to predict culture results of 97.9% (833 of 851). Unfortunately, such expertise is usually restricted to a venereal disease clinic, which may not be available to practicing physicians in the private sector. Consequently, the need exists for a rapid,

TABLE 2. *Results of Gram-stained smears and LAL assay for 550 male patients with culture-proven gonococcal and nongonococcal urethritis evaluated with the LAL test device and two lots of lysate*

Diagnosis	Lysate lot	No. tested	Gram-stained smears		LAL assay[a]	
			Positive	Negative	Positive	Negative
Gonococcal urethritis	XODY	212	203	9	209	3
	XOHA	154	150	4	154	0
Nongonococcal urethritis	XODY	113	0	113	4	109
	XOHA	71	1	70	2	69

[a] Incubation time was 30 min.

TABLE 3. *Clinical breakdown of urethral exudates from 550 men with gonococcal and nongonococcal urethritis*

Diagnosis	No. evaluated	% Urethral discharge		
		Profuse	Moderate	Scant
Gonococcal urethritis	366	40	40	20
Nongonococcal urethritis	184	10	51	39

accurate test which can differentiate between gonococcal and nongonococcal urethritis and does not require sophisticated methods and materials.

Recently, we reported the use of the LAL assay for the rapid, presumptive diagnosis of gonococcal and nongonococcal urethritis in men (17). The concept involved using a lysate made from the washed amoebocytes of the horseshoe crab (*Limulus polyphemus*) which forms a gel in the presence of small quantities (nanograms) of bacterial endotoxin. The lysate was shown to be extremely sensitive to intact outer membrane components of *N. gonorrhoeae* (13) and more sensitive to this organism than other gram-negative bacteria tested (18). When urethral exudates, properly diluted according to lysate sensitivity, were mixed with lysate and incubated, gelation occurred when *N. gonorrhoeae* was present. Gelation did not occur when the exudates were from patients with nongonococcal urethritis regardless of the many suspected causative agents of this disease, which include *Chlamydia trachomatis* and *Ureaplasma urealyticum* (3, 4, 14, 19). Sensitivity of the LAL assay was >99%, and specificity was >96%, with an overall accuracy in predicting cultures of >98%.

The results obtained in the incubation time study and LAL test device evaluation also show a high sensitivity and specificity. In the incubation time study, the time required for incubation was reduced from the standard 60 min to 30 min without significantly affecting the accuracy of the test. A sensitivity of 98.9%, a specificity of 97.6%, and an overall accuracy of 98.3% were obtained with samples from 301 patients by using the 30-min incubation time. The LAL test device demonstrated similar accuracy in 550 patients, with a sensitivity of 99.2%, a specificity of 96.7%, and an overall accuracy of 98.4%. This self-contained device permitted accurate and rapid dilution of the clinical specimen in a sealed system. Contamination of the sample with exogenous endotoxins and potential biohazards to the user were prevented. The relative ease of interpreting test results also allows utilization of the test by those who may be unskilled in laboratory techniques. With the LAL test device, physicians in the private sector could now obtain rapid results (within 30 min) with accuracy equal to that of Gram stain without the need for staining reagents, costly microscopes, and smear interpretations by trained microscopists. The six false-positive (3.3%) and three false-negative (0.8%) results obtained with the LAL test device would not affect the utility of the test. Although the reasons for these discrepancies are speculative, the possibility of sampling or technical error, contaminated (pyrogen-containing) equipment, or unreported antibiotic usage may provide an explanation.

The LAL assay as described is only applicable to urethral infections in which a urethral discharge can be demonstrated and at least 0.015 ml of exudate can be collected. This minimum volume was chosen since healthy males with no disease can usually strip their urethra, especially in the morning, and obtain a small amount of mucus (2). For patients who present with symptoms of urethritis from whom an adequate sample cannot be obtained, the private practitioner should refer them to a venereal disease clinic or other appropriate laboratory for Gram stain and culture. In cases of exudative urethritis where sufficient quantity of exudate can be collected, the LAL assay can be utilized, and appropriate therapy can be given during the patient's initial evaluation. The results obtained with the LAL test device suggest that additional studies be conducted in other clinics to thoroughly assess the value of this test in gonorrhea.

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