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Rapid Evaluation of Gonococcal and Nongonococcal Urethritis in Men with *Limulus* Amoebocyte Lysate and a Chromogenic Substrate

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A chromogenic substrate was used with *Limulus* amoebocyte lysate (LAL) and compared by parallel testing with the traditional gelation LAL method for the rapid evaluation of exudative urethritis in 125 male patients. Of these patients, 67 had positive cultures for *Neisseria gonorrhoeae* and 58 were negative. The corresponding prevalence of gonococcal urethritis was 53.6%. For assay, diluted urethral samples and chromogenic substrate were added directly to single-test LAL vials, and objective color endpoint determinations were made visually after a 10-min incubation period at 37°C. Sensitivity and specificity were 98.5% and 93.1%, respectively, with an overall accuracy in predicting culture results of 96.0%. The predictive value of a positive LAL test was 94.3% in our patient population; in a population with a prevalence of gonococcal urethritis of only 10%, the predictive value would be 61.3%. Results were not statistically different from those obtained by the 30-min gelation LAL method or by Gram-stained smears read by experienced microscopists ($P > 0.05$). Unlike the delicate gel, the color endpoint was not prone to accidental mechanical disruption during incubation or reading. Thus, use of a chromogenic substrate greatly improved the utility and speed of the LAL assay for evaluating men with exudative urethritis while not affecting the accuracy of the test.

Differentiation between gonococcal and nongonococcal urethritis is essential for the administration of appropriate therapy and proper disposition of sexual contacts. Currently, the Gram stain is the only widely accepted procedure used for the rapid evaluation of exudative urethritis, and in most cases a firm and immediate diagnosis can be made provided the smears are examined and interpreted by experienced microscopists (6). Unfortunately, such expertise is usually restricted to venereal disease clinics, which may not be available to private physicians, who see and treat most cases of urethritis (1, 3, 9). Moreover, without extensive experience in reading Gram-stained smears, the accuracy of the test decreases. In a recent report, Gram stain sensitivity of urethral smears varied from 79 to 96% and specificity from 52 to 97%, depending on the microscopist reading the smear (4). Consequently, the need exists for a simple, rapid, and accurate test which can differentiate between gonococcal and nongonococcal urethritis and can be used in the private practice setting.

Recently, we demonstrated the utility of the *Limulus* amoebocyte lysate (LAL) assay for the rapid, presumptive diagnosis of gonococcal and nongonococcal urethritis in men whereby positive or negative results were indicated by gelation or lack of gelation, respectively (13, 17, 18). We subsequently developed an LAL test device which could be used by those unskilled in laboratory procedures at the time of the patient's initial evaluation and reduced the time needed for incubation from 60 to 30 min while retaining an overall accuracy for predicting culture results of 98.4% (15). Other investigators have since used the LAL test device to evaluate exudative urethritis and have reported overall accuracies ranging from 94% (2) to 100% (10).

We report here the use of a chromogenic substrate with *Limulus* lysate for the rapid evaluation of men with exudative urethritis. Color development within 10 min was used as an objective visual endpoint. The chromogenic LAL method was comparatively evaluated with the gelation method and Gram stain for its ability to predict culture results for *Neisseria gonorrhoeae* in these patients.

MATERIALS AND METHODS

Patient population. A total of 125 men with uncomplicated exudative urethritis seen at the Columbus Health Department Venereal Disease Clinic were evaluated by both the 30-min gelation and 10-min chromo-

genic LAL methods. These patients had sought treatment because of urethral discharge or dysuria or both and were selected on a random basis. Demonstration of a urethral discharge at physical examination was considered as objective evidence of urethritis (8), and a minimum of 0.015 ml of exudate was needed for acceptance into the study. Patients who had received antibiotics within 10 days of presentation were excluded.

LAL test procedures. Urethral samples were collected and assayed with the 30-min gelation LAL method as previously described (15). Single-test LAL vials (Mallinckrodt, Inc., St. Louis, Mo.) with a minimum sensitivity of 0.1 ng of *Escherichia coli* endotoxin (lot EC-2; Bureau of Biologics, U.S. Food and Drug Administration, Bethesda, Md.) per ml were used for both the gelation and chromogenic LAL methods. The chromogenic substrate (lot R2FI) was kindly supplied by Don Mills of Mallinckrodt. Each vial contained 18.72 mmol of substrate and was reconstituted with 19.5 ml of sterile, pyrogen-free water. For assay, 0.25 ml of the diluted urethral sample was added to a single-test LAL vial, followed by the addition of 0.1 ml of chromogenic substrate. The vial was mixed and incubated at 37°C for 10 min in a heating block. After incubation, the vial was read visually by two separate observers for the development of a yellow color. Development of any yellow color when compared with a water blank was interpreted as a positive test; no color development was interpreted as a negative test. After visual reading, the reaction was stopped by the addition of 0.1 ml of 50% glacial acetic acid, and absorbance was measured at 405 nm in a spectrophotometer (model 330; Turner Associates, Palo Alto, Calif.) for correlation with the visual results. A lysate vial which contained substrate and pyrogen-free water served as a blank.

Comparative endpoint determinations between the gelation and chromogenic LAL methods were made, using an *E. coli* endotoxin (lot 16707; Mallinckrodt), and the results are shown in Table 1. The visual color endpoint was two twofold dilutions less sensitive than the gel endpoint, and the visual threshold correlated with an absorbance value of approximately 0.2. Clinical samples were diluted 1:1,600 for the gelation method as previously determined according to the lysate sensitivity (15) and 1:400 for the chromogenic method, assuming a collection of 0.025 ml of urethral exudate. The LAL test results were read without previous knowledge of the microbiological findings.

Clinical laboratory procedures. Urethral samples were also collected for Gram staining and for culture of *N. gonorrhoeae* on Martin-Lewis medium (State Health Laboratories, Columbus, Ohio). The diagnosis of gonococcal urethritis was based on a positive culture for *N. gonorrhoeae*. The details of the methods used and criteria followed for smear interpretation have been previously described (18). Cultures for viruses or chlamydiae were not done.

TABLE 1. Comparative reactions and endpoint determinations for the gelation and chromogenic LAL methods, using *E. coli* endotoxin

Endotoxin concn (ng/ml)	LAL method		A_{405}[c]
	Gelation[a]	Chromogenic[b]	
1	+	+	0.63 ± 0.03
0.5	+	+	0.37 ± 0.07
0.25	+	+	0.22 ± 0.03
0.13	+	−	0.10 ± 0.04
0.06	+	−	0.03 ± 0.02
0.03	−	−	0.01 ± 0.01

[a] Formation of an intact gel (+) or no gelation (−) after a 30-min incubation.

[b] Development of a visible yellow color (+) or no color (−) after a 10-min incubation.

[c] A_{405}, Absorbance value at 405 nm; mean of four determinations ± standard deviation.

Statistical analysis. The chi-square method of analysis was used for the determination of statistical significance. Sensitivity, specificity, and predictive values were computed according to the methods of Vecchio (21), assuming the culture method was 100% accurate. The predictive value of a positive LAL test was defined as the probability that a patient yielding a positive test actually has gonococcal urethritis for a given prevalence rate of the disease.

RESULTS

The results obtained with the Gram stain and both LAL methods for 125 patients with exudative urethritis are shown in Table 2. Of the 125 patients, 67 had culture-proven gonococcal urethritis and 58 had nongonococcal urethritis. The sensitivities and specificities were 95.5% (64 of 67) and 100% (58 of 58), respectively, for the Gram stain and 98.5% (66 of 67) and 93.1% (54 of 58), respectively, for each LAL method. Overall ability to predict culture results was 97.6% (122 of 125) for the Gram stain and 96.0% (120 of 125) for each LAL method. There were no statistical-

TABLE 2. Results of Gram-stained smears and both gelation and chromogenic LAL methods for 125 male patients with gonococcal and nongonococcal urethritis

Diagnosis	No. of patients	Gram-stained smears		LAL assay method			
				Gelation[a]		Chromogenic[b]	
		Positive	Negative	Positive	Negative	Positive	Negative
Gonococcal urethritis	67	64	3	66	1	66	1
Nongonococcal urethritis	58	0	58	4	54	4	54

[a] Urethral samples diluted 1:1,600; incubation time, 30 min.

[b] Urethral samples diluted 1:400; incubation time, 10 min; results based on visual readings.

ly significant differences between the LAL methods and Gram stain results ($P > 0.05$).

The correlation between the absorbance values and visual test results for urethral samples from patients with gonococcal and nongonococcal urethritis which were assayed by the chromogenic LAL method is shown in Fig. 1. The objectivity of reading the LAL test visually is demonstrated since results matched absorbance values obtained with the spectrophotometer, using a threshold value for a positive test of 0.22 (Table 1). The absorbance values for positive and negative visual test results were 0.90 ± 0.18 and 0.06 ± 0.05 (mean $\pm$ standard deviation), respectively. The lowest absorbance value obtained for a sample read visually as a positive test was 0.3. Positive and negative results were easy to differentiate visually, and the same results were obtained by both observers.

The prevalence of gonorrhea in men evaluated during the study was 53.6% (67 of 125), and the predictive value of a positive test for both LAL methods was 94.3%.

DISCUSSION

The mechanism of gel formation in the LAL assay is enzymatic in nature and involves a proclotting enzyme and coagulogen, two proteins that are contained in *Limulus* lysate. Initially, the proclotting enzyme is activated by bacterial endotoxin (19), although a factor B now appears to be involved indirectly (12). Once activated, the proclotting enzyme splits the protein coagulogen into peptides which interact to form a clot (20). Recently, it was reported that the proclotting enzyme has amidase activity and will cleave certain synthetic amino acid substrates which act as carriers for a chromogenic *p*-nitroanilide group (11). Thus, by using a chromogenic substrate, the subsequent splitting of coagulogen by the activated proclotting enzyme and formation of a gel as an endpoint could be eliminated. Moreover, intensity of the color generated is proportional to the quantity of endotoxin present (5). Consequently, this procedure has been evaluated for the quantitation of endotoxin (16) and for the detection of endotoxin in blood (22).

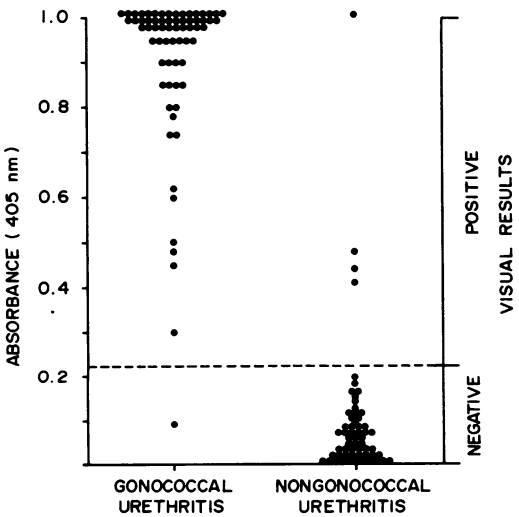

FIG. 1. Comparison of absorbance values with visual results for urethral samples from 67 male patients with gonococcal urethritis and 58 patients with nongonococcal urethritis which were assayed by the 10-min chromogenic LAL method.

In the study reported here, we used a chromogenic substrate with *Limulus* lysate for qualitative (color versus no color) determination of endotoxin in samples of urethral exudate for differentiation between gonococcal and nongonococcal urethritis in men. The simplicity of performing the 30-min gelation LAL assay was retained with the chromogenic method. The only modification was the addition of substrate to the LAL vial, a process which can be incorporated in the manufacture of the single-test vials. Use of acetic acid to stop the color reaction after the 10-min incubation period would not be necessary. However, by adding acetic acid to the LAL vial, a permanent record of the patient's test results can be made. Incubation time was reduced from the 30 min required for gelation to 10 min; and, unlike the delicate gel, the color endpoint was not prone to accidental mechanical disruption during incubation or reading. Moreover, there was a wide range around the visual threshold (Fig. 1) which would allow for a margin of error in the volume of sample collected. This margin of error was also shown in quantitative LAL studies with the gelation method whereby a 50% change in sample volume would result in less than a 4% change in sensitivity at the breakpoint dilution (14).

The use of a chromogenic substrate as an alternate to gel formation with color development as an objective endpoint greatly improved the utility and speed of the LAL assay in evaluating men with exudative urethritis. The overall accuracy of 96.0% was equivalent for both the chromogenic and gelation LAL methods used in this study and was not statistically different ($P > 0.05$) from the accuracy of 98.4% previously reported in evaluating 550 patients with the 30-min gelation procedure (15). The predictive value of a positive test was also high (94.3%) for the prevalence of gonorrhea (53.6%) encountered in this study. However, in private practice settings, the relative prevalence of gonorrhea among men with acute urethritis would be about 30% (7). The predictive value of a positive LAL test at this lower frequency of gonorrhea would

be 86%, which is sufficiently high that a patient with a positive test may be treated for gonorrhea. However, the possibility exists that the prevalence of gonococcal urethritis may be as low as 10% (7), and the predictive value of a positive test would then be 61.3%. Therefore, it is recommended that clinicians be familiar with the prevalence of gonococcal urethritis in their patient populations when interpreting LAL test results. Only five (4%) LAL test results did not correctly predict culture results. Although the reasons for these discrepancies are speculative, the possibility of culture failures, vancomycin-susceptible strains, technical error, contaminated (pyrogen-containing) equipment, or unreported antibiotic usage may provide an explanation.

Thus, by modifying the LAL assay with the addition of a chromogenic substrate, physicians could obtain objective test results within 10 min of sample collection with accuracy equal to Gram stain smears read by experienced microscopists and to cultures processed by venereal disease clinics without the need for sophisticated methods and materials.

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