Reactivity of the limulus lysate assay with uterine cervical secretions
A preliminary evaluation

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SUMMARY A limulus lysate assay was performed on cervical secretions from 66 women. When secretions were tested at a 1/100 dilution the assay gave a positive result in 15 (62.5%) of 24 patients with gonorrhoea confirmed by Gram-stained smear or culture or both. When secretions from seven of the nine remaining patients who had gonorrhoea but negative results to the limulus lysate test were retested at a 1/50 dilution, two gave a positive result, increasing the positivity rate of the test to 17 (70.8%) of 24 infected patients. Material from one patient with a history of contact with gonorrhoea and from three (7.3%) of the other 41 patients without any history of gonorrhoea gave positive reactions.

Introduction

Despite improvements in the cultural diagnosis of gonorrhoea1 2 and the advent of several transport and growth systems,3 4 problems associated with maintaining the viability of the gonococcus still exist in certain areas. Gram-staining of material from the genitourinary tract is the only widely accepted non-cultural method for the diagnosis of gonorrhoea. The reliability of Gram-staining for male cases is high; an unequivocally positive or negative result for smears of urethral discharge provides an immediate differential diagnosis between gonococcal and nongono- coccal urethritis in 85% of patients.5 However, in female cases Gram-staining of urethral and cervical material will detect only 55-65% of patients from whom Neisseria gonorrhoeae is subsequently isolated by culture.6 8

An assay for endotoxin resulted from the finding by Levin and Bang9 that a lysate of washed amoebocytes of the horseshoe crab (Limulus polyphemus) formed a gel in the presence of minute amounts of endotoxin elaborated by Gram-negative bacteria. Since the demonstration by Rice and Kasper10 of the sensitivity of the limulus endotoxin assay for components of N gonorrhoeae, the system has been shown to be of value in the rapid presumptive diagnosis of gonococcal urethritis in men.11 12 Because of the lack of sensitivity of Gram-staining in female patients, any new non-cultural diagnostic method is of greater potential value in the diagnosis of gonorrhoea in women than in men. For these reasons we considered it worthwhile to report our preliminary evaluation of the limulus lysate assay when applied to cervical secretions.

Patients and methods

STUDY POPULATION AND DIAGNOSIS
Sixty-six women consecutively attending the department of genitourinary medicine at the Black Street Clinic, Glasgow, were investigated. Specimens were taken from the urethra and cervix for microscopical and cultural examination and from the rectum for culture only. Cultural and identification methods for N gonorrhoeae were as described.13

COLLECTION OF SECRETIONS
The cervix was cleaned with a cotton-wool swab held in sponge-holding forceps under direct vision.14 Secretions were collected from the endocervical canal by gentle aspiration through a sterile polythene capillary tube (chromatography column tubing, internal diameter 1.0 mm, obtained from Pharmacia Fine Chemicals, Uppsala, Sweden), one end of which had been inserted through the os to about 1 cm; the other end of the tube was attached to a 5-ml syringe.

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containing 1 ml pyrogen-free water. Secretions in the tubing were ejected into a pyrogen-free plastic container. The secretions were stored at -20°C until required.

**LIMULUS ASSAY**
Frozen specimens of cervical secretions were thawed, mixed thoroughly, and diluted in pyrogen-free water to a final dilution of 1/100; 0.1 ml of the diluted secretion was mixed in a pyrogen-free plastic container with 0.1 ml of reagent reconstituted from the 50 Test Pyrotest™ vial (Difco Laboratories, Detroit, Michigan), incubated at 37°C in a water bath for one hour and read. Results were interpreted in accordance with the manufacturer’s instructions; a firm opaque gel which remained adherent to the bottom of the vial when inverted through 180° was scored as positive; the absence of a firm gel was scored as negative. Control samples with known positive and negative results were tested with each batch of assays. The sensitivity of the limulus assay was demonstrated by the detection of 0.125 µg/l Difco Pyrotol positive control Escherichia coli endotoxin.

The limulus assays were read without previous knowledge of the conventional microbiological results. After the correlation between the limulus assay results and a diagnosis of gonorrhoea had been made, certain specimens showing an apparently false-positive limulus result were retested at a dilution of 1/200 while specimens showing a false-negative result were retested at a dilution of 1/50.

**PROTEIN ESTIMATION**
To determine whether false-negative results could possibly be due to the secretions being very dilute, the protein concentration of several secretions (four specimens with false-negative and five with confirmed positive results chosen at random) was determined.14

**STATISTICAL ANALYSIS**
The $\chi^2$ test with Yates’s correction was used to test the correlation between limulus assay results and conventional microbiological findings. Student’s $t$ test was used to compare the mean protein concentrations in secretions giving false-negative and confirmed positive limulus results.

**Results**

**CULTURE AND MICROSCOPY**
The results of culture and microscopy for *N* gonorrhoeae were shown in relation to limulus lysate assay results in the table. The limulus assay gave a positive result in 19 (28.8%) of the 66 patients investigated. The result was reactive in 15 (62.5%) of the 24 patients with cervical gonorrhoea (positive smear or culture result or both) compared with only four (9.5%) of the 42 patients without any microbiological evidence of gonorrhoea. This difference is statistically highly significant ($\chi^2$ 18.4; p<0.001). When secretions from seven of the nine patients with gonorrhoea but negative limulus assay results at a dilution of 1/100 were retested at 1/50 two gave a positive result and this increased the positivity rate of the test to 17 (70.8%) of 24 infected patients.

**PRESENTING DIAGNOSES**
The presenting diagnoses (multiple in a few cases) of the 42 patients in whom there was no microbiological evidence of gonorrhoea were as follows: *Chlamydia trachomatis* infection, eight; trichomoniasis, 10; candidosis, eight; warts, five; and no infection detected, nine. The remaining eight patients, all with negative limulus assay results, had been treated for gonorrhoea within the preceding three months (range one week to three months).

The limulus assay gave a positive result in one patient who was a contact of gonorrhoea, one patient from whom *C* trachomatis was isolated, one patient with warts, and one patient in whom no abnormality was detected. If the patient who was a contact of gonorrhoea is excluded the presumed false-positivity rate is reduced to 7.3%; on retesting secretions from two of the three remaining patients at a dilution of 1/200 both gave negative results, reducing the false-positivity rate to 2.4%.

**Table. Results of conventional microbiological investigations for Neisseria gonorrhoeae and of the limulus lysate assay for endotoxin in cervical secretions from 66 women**

<table>
<thead>
<tr>
<th>Microbiological results</th>
<th>No of patients</th>
<th>Results of limulus lysate assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Culture +/smear +</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>Culture +/smear -</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Culture −/smear +</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Culture −/smear −</td>
<td>42</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>66</td>
<td>19</td>
</tr>
</tbody>
</table>

+ Positive  – negative
MEAN PROTEIN CONCENTRATION

The mean protein concentration in the secretions from four patients with gonorrhoea who gave negative limulus results was 0.42 g/l compared with a mean concentration of 1.50 g/l in the secretions from five patients with gonorrhoea who gave positive limulus results. The difference between the two means is statistically significant (t 10.12; p<0.001).

Discussion

Although the number of infected patients was low there was a highly statistically significant correlation (p<0.001) between limulus lysate assay results and conventional microbiological evidence of gonorrhoea. The limulus assay result was positive in only 62-5% of women with cervical gonorrhoea and was less sensitive than Gram-staining, which detected 75% of infected women. However, the positivity rate of 62-5% for the limulus test is within the usual range (55-65%) of sensitivity found by Gram-staining.6-8

In the study of Spagna et al11 the limulus assay applied to urethral exudates from men gave a positive result in all 73 culture-positive cases of gonorrhoea tested. Our finding that the positivity rate for the limulus test could be increased to 70-8% by retesting limulus-negative secretions from patients with gonorrhoea at a dilution of 1/50, combined with the lower mean protein concentration found in secretions from such patients, suggests that variation in sampling may be the main reason for the poorer results in women. This variation could possibly be overcome by standardising the test on the basis of a determined protein concentration, for example, rather than by testing all specimens at a fixed dilution. A microdilution technique has also been reported to improve the sensitivity of the limulus assay.12

Positive limulus test results were considered to be unrelated to gonococcal infection in three (7.3%) of 41 patients. It is possible that these patients may have had a recent gonococcal infection, of which we were unaware. However, the limulus assay gave a negative result in all eight patients treated for gonorrhoea within the preceding three months (less than three weeks in four patients) suggesting that gonococcal components reactive in the assay are eliminated from the cervix in a fairly short time.

Clearly, the specificity of the limulus assay requires further evaluation on a larger series of women. In particular, it would be of value to combine these studies with quantitative aspects of the "commensal" flora of the female genital tract to determine the extent of interference due to cervical colonisation with coliform organisms and other endotoxin-positive bacteria. In men the situation is quite different; the urethra is normally free from heavy colonisation with endotoxin-positive bacteria and, as shown by Spagna et al,11 the gonococcus is almost invariably the cause of a positive limulus assay result for endotoxin.

Recently these workers15 extended their studies on the limulus assay to detect gonococcal endotoxin in cervical exudates diluted 1/800. The assay result was reactive in 17 (94%) of 18 of infected women, all of whom had a purulent cervical discharge. The lack of such a discharge in most of our infected patients might explain the lower reactivity rate in our study. Spagna et al13 noted that four of eight patients with nongonococcal cervicitis, in whom other Gram-negative bacteria were present, gave false-positive limulus assay results when exudates were tested at a 1/200 dilution: all eight results were negative when exudates were tested at a 1/800 dilution. Our combined findings suggest that it may prove difficult to obtain reliable results when exudates from all patients, irrespective of the presence or absence of a purulent cervical discharge, are tested at the same dilution.

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References

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