UNUSUAL PLEUROPNEUMONIA-LIKE ORGANISMS ISOLATED IN A STUDY OF TRICHOMONAS VAGINALIS FROM CASES OF CHRONIC URETHRITIS

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Recently a study was undertaken to ascertain the bacterial flora of the male urethra and the relationship of Trichomonas vaginalis and pleuropneumonia-like organisms (PPLO) in male patients with chronic urethritis. The PPLO encountered in this study differed from those described in the literature in that they could not be isolated on PPLO agar (Difco) supplemented with PPLO serum fraction A. Isolation was accomplished on beef heart infusion agar (Difco) supplemented with 5 per cent defibrinated horse blood. Because of these findings it is important to inform those workers engaged in the laboratory isolation of PPLO that some strains may be overlooked due to their unusually fastidious growth requirements and when relying entirely on PPLO agar supplemented with serum fraction A. The morphology of the colonies, as influenced by type of medium, is also illustrated.

MATERIALS AND METHODS

The data were obtained from a study of 36 urethral discharge specimens taken from 34 men presenting themselves for examination at the urology clinic at Jefferson Medical College Hospital. These patients presented no evidence of venereal disease but had returned to the clinic frequently for a period of 2 to 17 months with a persistent urethral discharge. Therapy was initiated after their first visit and various antibiotics, washings, and soaks were employed with these patients. The specimens were taken either at the first visit to the clinic before therapy or after subsequent visits.

The specimens were obtained after the penis and meatus were cleansed thoroughly with 70 per cent alcohol and allowed to air dry. The penis was stripped and a drop of discharge was collected on two sterile cotton swabs. A wet mount was made immediately with the material from one swab and examined for trichomonads. The second swab was placed in 3 ml of PPLO broth (Difco) (Morton et al., 1951) and transported to the laboratory. Examination was begun within 3 hr of the time the specimen was taken.

In the laboratory the swab was removed by squeezing it along the side of the tube and 0.1 ml of the broth was pipetted to each of the following media: blood agar base (BAB, Difco) containing 5 per cent defibrinated horse blood incubated aerobically and anaerobically; PPLO agar (Difco) containing 1 per cent PPLO serum fraction A, (Smith and Morton, 1951) incubated aerobically and anaerobically; and PPLO agar (Difco) plus 1 per cent serum fraction A with thallium acetate added to make a final concentration of 1:1000 (Morton and Lecce, 1953). The aerobic cultures were incubated at 37 C and examined at 24 and 48 hr and at 5 days. The anaerobic cultures were incubated at 37 C in an atmosphere of 10 per cent carbon dioxide (Morton, 1943) and examined at 48 hr. All cultures were examined under a dissecting microscope (25 ×) with oblique illumination. All different colony types were examined by smear and subcultured to appropriate media for identification. Cultures on PPLO medium were examined under low power of the microscope (100 ×). PPLO colonies were identified by their typical morphology, by their exacting requirements for serum protein, by their inability to be removed with an inoculating needle, and by their staining reaction (Dienes and Weinberger, 1951).

RESULTS

Bacterial, PPLO and Trichomonas isolations. Trichomonas vaginalis was demonstrated in the
table 1

Relationship of bacterial flora to Trichomonas

<table>
<thead>
<tr>
<th></th>
<th>Trichomonas Positive 35</th>
<th>Trichomonas Negative 11</th>
<th>Total 46</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus albus</em></td>
<td>17*</td>
<td>4</td>
<td>21</td>
</tr>
<tr>
<td><em>Staphylococcus citreus</em></td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Streptococci</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Various species)</td>
<td>5</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Diptheroids</td>
<td>8</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>3</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Proteus species</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>Monilia albicans</em></td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>PPLO</td>
<td>4</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>No bacteria</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

* Number of specimens from which the bacteria were isolated.

urethral strppings from 25 of the 34 patients selected for these studies. The clinical interpretation and correlation studies are the subject of the article by Feo et al. (1956). The bacterial flora consisted of, in order of predominance, *Staphylococci*, *diphtheroids*, *streptococci*, *Escherichia coli*, *Proteus species*, and *Monilia albicans*, as is shown in table 1. PPLO were isolated from 5 specimens.

Relationship of PPLO to Trichomonas. PPLO were isolated from 5 of the 34 patients studied or 14.7 per cent (table 2). From the 25 patients with trichomonads, PPLO were isolated for 4 or 16 per cent. In the 11 specimens in which trichomonads were not demonstrated only one strain of PPLO could be identified. It was of interest to determine if a similar relationship existed between PPLO and trichomonads as was shown to exist between *Neisseria gonorrhoeae* and PPLO (Somerson et al. 1955).

With such a small population the Yates correction is necessary, which produces a Chi square equal to 0.0304. This means that this particular distribution would arise through chance eight times out of ten. From the data taken on the basis of a Chi square analysis, (Quenouille, 1950), there appears to be no association in this population, i. e., no evidence is found that PPLO coexists with trichomonads.

Isolation of unusual PPLO. The 5 strains of PPLO isolated would have been overlooked if blood agar plates had not been inoculated with the same specimens. On primary isolation plates a glimmering effect, when the blood plates were examined under oblique illumination, attracted the investigator's eye. No colonies could be observed when examined directly or with the dissecting microscope.

Because of the blood it was difficult to see unstained colonies even with the low power of the microscope. By placing a drop of Dienes stain on the agar surface and examining with the low power of the microscope colonies of typical PPLO were observed (figure 1). The corresponding initial agar plates of these specimens were examined closely but colonies of PPLO were not observed even after three blind transfers. Blood agar blocks of the initial isolates were transferred to PPLO agar, incubated aerobically at 37 C and examined after 48 and 72 hr and again after 5 days. Colonies could only be observed in the area of diffusion around the blood agar block. How-

![Figure 1. Initial PPLO colony on blood agar stained with Dienes' stain (magnification 1000 ×).](http://jb.asm.org/)
However, the colonies that grew in the area of diffusion appeared as large lacy vacuolated colonies when examined with the low power microscope (figure 2). These lacy colonies could be transferred without any accompanying bacterial colonies. After four transfers on PPLO agar from blood agar these strains grew sparsely over the entire agar surface and were not confined to the area of diffusion. The colonial morphology of these colonies was lacy and vacuolated and twice the size of the typical colony. The lacy colonies could be transferred to blood agar and typical colonies would result. After 6 months, during which transfers were made at 4- to 5-day intervals, these strains lost the lacy, vacuolated appearance on PPLO medium and displayed the familiar fried egg morphology (figure 3).

Within the first several weeks after isolation various growth characteristics of these strains were tested. They were capable of growing optimally when either 1,000 units of penicillin per ml or a final dilution of 1:1000 thallium acetate, separately or together, were added to 5 per cent blood agar. They could not be removed from the agar surface with an inoculating needle. They required the addition of animal protein to the media for growth. It was found that as little as 0.1 per cent defibrinated horse blood added to BAB or PPLO agar would support growth. The initially isolated strains did not grow when serum fraction A, hemoglobin (Difco), or hemolysate (2 per cent packed blood cells lysed with sterile distilled water) were added to the media at 1 per cent levels. However, at 3 and 5 per cent levels of these supplements a few lacy vacuolated colonies did appear.

It was found that medium with a pH of 7.0 and an agar concentration of 1.2 per cent produced a granular colony. Higher pH levels and higher agar concentrations resulted in lacy vacuolated colonies.

**DISCUSSION**

The bacterial species were isolated with equal frequency from those specimens showing trichomonads as from those without trichomonads, which was suggestive that trichomonads, rather than the bacteria, might be a causative agent of the chronic urethritis.

The percentage of PPLO isolations, 14.7 per cent, is considerably lower than the incidence of 86 per cent reported isolated from the uterine cervix by Rubin et al. (1954) and somewhat lower than the 20 per cent reported from the male urethra by Morton et al. (1951). This lower incidence could possibly be due to the various antibiotic therapies given to these patients prior to collecting the initial specimen.
Although the sample is small, it appears, statistically, extremely unlikely that PPLO and trichomonads coexist in these cases of chronic urethritis.

The 5 strains of PPLO isolated in this study show unusual growth characteristics and would have been overlooked if blood agar media had not been employed. These unusual strains showed typical colonial morphology on blood agar media. They did not grow initially on PPLO agar but, when they became adapted to PPLO medium, they presented a lacy, vacuolated colony approximately twice the size of the colonies when grown on blood agar. The growth of lacy, vacuolated colonies in the area of diffusion surrounding the block of blood agar containing the inoculum on PPLO medium indicates that either the PPLO medium is toxic to certain strains or that it lacks certain nutrients which are contained in whole blood. The addition of hemoglobin, hemolysate, serum fraction, cholesterol, or lecithin did not indicate which components in whole blood allowed typical growth to take place. The pH and agar concentration of the medium appear to have an effect on the morphology of the colonies in that the higher the pH and the higher the agar concentration the more the colonies were prone to be the lacy, vacuolated type. These strains appear to be more fastidious in their growth requirements than strain 48 which has been used by Smith and Morton (1952), and Smith et al. (1954) in metabolism experiments. We are unable to explain why these strains failed to grow initially on PPLO medium. For the present it appears that the presence of blood in the medium inoculated with clinical material enhances the chance of isolating this type of PPLO.

SUMMARY

Pleuropneumonia-like organisms (PPLO) were isolated from 5 (14.7 per cent) of the 34 patients studied. These strains were unusual in that they were isolated from blood agar medium and did not grow initially on supplemented PPLO agar. When adapted to PPLO medium, these colonies displayed a lacy vacuolated colonial morphology. PPLO and trichomonads were not associated in these patients with any statistical significance.

REFERENCES