Comparison of Cultural and Immunofluorescent Procedures in the Identification of *Haemophilus vaginalis*

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SUMMARY

The application of immunofluorescent techniques for the detection and identification of *Haemophilus vaginalis* in vaginal secretions by using fluorescent *H. vaginalis* antiglobulin (strains nos. 317, 4984) were found to be as specific and sensitive as cultural methods and had the advantage of being simple and rapid.

INTRODUCTION

Several investigators (Leopold, 1953; Gardner & Dukes, 1954, 1955; Gardner, Dampeer & Dukes, 1957; Amies & Jones, 1957; Brewer, Halpern & Thomas, 1957; Edmunds, 1960; Lapage, 1961) have reported the occurrence of a small, Gram-negative bacillus in the male and female genitro-urinary tract. These investigators used various media for the isolation and cultivation of this organism, named *Haemophilus vaginalis* by Gardner & Dukes (1955). Although most of the investigators used the criteria for identification established by Gardner & Dukes, several of the reports varied as to the cultural behaviour and staining characteristics. The organism was regarded as quite fastidious. In view of these problems, it was decided to compare immunofluorescent procedures, which had been found to be highly specific (Redmond & Kotcher, 1963) with cultural procedures, Gram-stained smears, and 'clue-cells' in the isolation and identification of this organism from clinical material at Houston, Texas and Louisville, Kentucky, U.S.A.

METHODS

Media. In the cultural procedures used in Louisville the following media were employed for the isolation of organisms from vaginal swabs which had been put into proteose peptone no. 3 (Difco) carrying broth; Casman agar with 5% sterile, defibrinated rabbit blood; Rogosa agar; blood agar (human out-dated blood); eosin-methylene blue agar; GC medium (Difco); PPLO broth and agar (Difco); National Institutes of Health thioglycollate with and without 5% horse serum; and Douglas broth.

In the cultural procedures used at Houston, Brewer thioglycollate broth without methylene-blue indicator (Baltimore Biological Laboratories) was used as the transport medium for clinical specimens. Casman rabbit blood agar was used for isolation. Any organism that could not be identified from this primary culture was subcultured on special media for identification. In both laboratories (Louisville, Houston) the Casman plates were cultured in a candle jar at 37°. The vaginal swabs in transport medium were streaked on cultivation media within 2-6 hr. after having

been obtained from the patient. The criteria for the identification of *Haemophilus vaginalis* were those given by Dukes & Gardner (1961).

Staining methods. The staining methods, immunization of rabbits, the method of conjugating fluorescein isothiocyanate to the globulin from the antiserum of immunized rabbits, and the immunofluorescent procedures were as described by Redmond & Kotcher (1963).

Clinical methods. Each patient was examined for symptoms of vulvitis, vaginitis and cervicitis by a gynaecologist. Vaginal secretion smears were made on three microscope slides and a vaginal swab was inoculated into a transport medium. A drop of vaginal secretion was examined by wet-mount for the presence of 'clue cells', leucocytes, *Trichomonas vaginalis*, and yeast-like organisms. One vaginal smear was Gram-stained and examined for the presence of small Gram-negative bacilli and 'clue cells'. A second vaginal smear was fixed in absolute methanol for 5 min., air dried, and stained with fluorescent *Haemophilus vaginalis* antiglobulin by the direct method. This smear was examined for fluorescent bacteria. The third vaginal smear was fixed in absolute methanol and held in reserve.

Table 1. *Comparison of four methods in studying Haemophilus vaginalis in three groups of patients*

Number of patients and group			Method			
27 private patients (Houston, Texas)	54 private patients (Louisville, Kentucky)	51 clinic patients (Louisville, Kentucky)	Culture	Fluorescent antibody	Clue cell*	Gram stains
14	3	5	+	+	+	+
0	0	0	+	+	+	-
0	0	0	+	+	-	-
0	0	0	+	-	-	-
3	3	1	-	-	-	-
0	1	1	-	+	+	+
0	1	2	-	-	+	+
5	36	38	-	-	-	+
0	0	0	+	-	+	+
4	5	3	+	+	-	+
0	0	0	-	+	+	-
0	0	0	-	+	-	-
0	1	0	-	-	+	-
0	0	1	+	-	-	+
1	4	0	-	+	-	+
0	0	0	+	-	+	-

* Clinic patients were examined for clue cells by Gram-stained smears rather than by wet mount.

RESULTS

A comparison of four methods for studying *Haemophilus vaginalis*, namely, culture, FA staining, presence of 'clue cells', and Gram reaction is presented in Table 1. FA staining revealed the presence of *H. vaginalis* in five patients from whom the organism was not cultured. In no instance was the organism cultured and not found on vaginal smears by FA staining. The 'clue cell' and the Gram-reaction observations are incorporated in Table 1 although it is realized that these techniques are not specific for *H. vaginalis*.

The incidence of *Haemophilus vaginalis*, as revealed by FA staining, accepted cultural procedures and by both methods in the same three groups of patients is presented in Table 2. The Houston patients were highly selected because it was decided to use such a group as a control to determine the sensitivity and specificity of the methods. No such selection was carried out with either group at Louisville.

Table 2. *Incidence of Haemophilus vaginalis by fluorescent antibody and cultural methods*

Number of patients and group	Positive culture		Positive fluorescent antibody		Positive culture and fluorescent antibody	
	No.	%	No.	%	No.	%
27 private patients, Houston, Texas	19	70	19	70	19	70
54 private patients, Louisville, Kentucky	8	15	13	24	8	15
51 clinic patients, Louisville, Kentucky	9	18	9	18	8	16

A great variety of organisms may be found in the vagina, particularly in patients with vaginitis, cervicitis and vulvitis. In Table 3 a list is given of other organisms found in association with *Haemophilus vaginalis* in the three groups of patients examined. There was a considerable difference in the frequency with which certain organisms, such as *Staphylococcus aureus*, *Streptococcus viridans*, *Micrococcus* sp., *Escherichia coli*, diphtheroids, and Doederlein's bacillus were isolated as between the Houston patients and the Louisville patients. However, different cultural procedures were used and different bacteriologists were involved in studying the patients of the two cities.

Table 3. *Other organisms found in association with Haemophilus vaginalis in vaginal secretions*

Organisms associated with <i>Haemophilus vaginalis</i>	Number of patients and group		
	19 private patients (Houston, Texas)	13 private patients (Louisville, Kentucky)	10 clinic patients (Louisville, Kentucky)
<i>Trichomonas vaginalis</i>	3	2	3
<i>Candida</i> sp.	1	1	0
<i>Staphylococcus aureus</i>	1	5	5
<i>Streptococcus viridans</i>	8	1	0
<i>S. anhemolyticus</i>	0	1	0
<i>S. hemolyticus</i>	0	2	0
<i>Enterococcus</i>	0	2	0
<i>Sarcina</i> sp.	0	3	1
<i>Micrococcus</i> sp.	6	0	0
<i>Escherichia coli</i>	0	3	1
Diphtheroids	16	1	4
Doederlein's bacillus	0	10	4
No organism	1	0	2

The complete flora studies on the Louisville, Kentucky, groups were done by Miss Carolyn Frick. The flora studies on the Houston, Texas group were done by Mrs Martha Foster.

DISCUSSION

In interpreting the results of the three patients groups, the Houston group was considered as the control group. The clinician selected the patients by clinical signs and symptoms of *Haemophilus vaginalis* vaginitis, and also included normal women, and women with vaginitis caused by organisms other than *H. vaginalis* in this group. The two groups of patients from Louisville were randomly selected patients; however, some patients from the private practice group were suspected by clinical observation of having *H. vaginalis* vaginitis.

The fluorescent *Haemophilus vaginalis* antiglobulin was as sensitive as cultural techniques in identifying *H. vaginalis*. In the clinical specimens examined, FA methods revealed six additional specimens (five patients) with *H. vaginalis* which was not isolated by cultural methods. Non-specific fluorescence was ruled out by subjecting the other species of bacteria isolated to staining with fluorescent *H. vaginalis* antiglobulin by the direct immunofluorescent method. In a single case from the Louisville gynaecology clinic, *H. vaginalis* was isolated by cultural methods, whereas the vaginal smear from this patient was negative by direct immunofluorescent reaction. However, the culture of *H. vaginalis* was positive for fluorescence by the direct immunofluorescent reaction.

Because of the fastidious nature of *Haemophilus vaginalis*, it is necessary to use a highly enriched culture medium, such as Casman rabbit blood agar, for the isolation of the organism. On this medium other organisms from the vagina grow rapidly and may, on occasion, overgrow *H. vaginalis*. In cultivating this fastidious bacillus from clinical specimens it is important that the specimens do not remain in transport media more than 6–8 hr. or the organisms will not survive. Because of these cultural and transport difficulties, it is believed that FA techniques are more practical in the identification of *H. vaginalis* particularly as in this investigation the use of fluorescent *H. vaginalis* antiglobulin was as specific and somewhat more sensitive than culture methods. Also, it has the advantage of simplicity and rapidity, as well as not requiring the processing of the clinical specimen immediately, which is necessary with cultural procedures.

The difference in the percentage incidence of *Haemophilus vaginalis* in the Houston patients as compared with the Louisville patients is explained by the fact that the Houston gynaecologist selected his patients for *H. vaginalis* vaginitis. The two Louisville groups were unselected, and the incidence in this series of patients was comparable to that found by Gardner *et al.* (1957), Ritzerfeld & Kummel (1960), Kummel & Ritzerfeld (1961), and Wurch & Lutz (1955). The incidence of *H. vaginalis* in Louisville, however, was lower than that reported by Brewer *et al.* (1957), Edmunds (1959) and De La Fuente, Rico & Soria (1959).

Although Gardner & Dukes (1955) recommended the wet mount for the detection of 'clue cells', other investigators (Edmunds, 1959; Lapage, 1961) have also used the Gram-stained vaginal smear. In the present work, patients from the Louisville clinic group were examined for 'clue cells' by Gram-stained smears. Small Gram-positive organisms were frequently found attached to the epithelial cells, thus producing the same phenomenon in wet mounts as when *Haemophilus vaginalis* was attached to the cells. In the two Louisville groups, 'clue cells' were present when cultural and immunofluorescent methods did not reveal *H. vaginalis* (Table 1);

it was also noted that *H. vaginalis* was found when 'clue cells' were absent. LaPage (1961) observed Gram-positive and Gram-negative mixed 'clue cells', thus casting doubt on the significance of the association of such cells with the isolation of the small Gram-negative bacillus.

Comparison of the organisms found in association with *Haemophilus vaginalis* in the vaginal secretions of the three groups studied showed a marked difference as between the Houston group and the two Louisville groups (Table 3). The flora studies of the three groups were not made with identical transport and cultural media, nor were the bacteriologists the same. These technical differences are probably the most satisfactory explanation for the differences observed in the vaginal floras. The absence of Doederleins' bacillus from the Houston group and its presence in the two Louisville groups is the most striking feature. However, Rogosa medium or tomato juice agar medium was not used in studying the Houston group, and for this reason Doederlein's bacillus may have been missed.

In comparing the two Louisville groups, Doederlein's bacillus and *Staphylococcus aureus* were the organisms most frequently associated with *Haemophilus vaginalis*. *Trichomonas vaginalis* was associated with *H. vaginalis* in 18 % of the Louisville private practice group, 28 % of the Louisville clinic group, and 16 % of the Houston private practice group. Diphtheroids were frequently isolated from the Louisville clinic group and the Houston private practice group; however, they are considered to be part of the normal vaginal flora. Brewer *et al.* (1957), Heltai (1959), Edmunds (1959), and LaPage (1961) stated that *H. vaginalis* was isolated more frequently with a mixed microbial flora. In the present work too, more often than not, *H. vaginalis* was isolated together with a mixed flora. The frequently observed pure cultures of *H. vaginalis* reported by Gardner & Dukes (1955) were noted in only three instances out of 122 patients in the present work.

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