IDENTIFICATION OF *HAEMOPHILUS VAGINALIS*¹

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During the period 1953 to 1955, several publications reported the isolation of small, gram-negative, hemophilic bacteria from the genitourinary tract. Leopold (1953) isolated the bacterium, gave a partial description, and suggested a relationship to the genus *Haemophilus*. Independently, Gardner and Dukes (1955) reported the occurrence of the organism and elaborated the clinical condition of vaginitis with which it is associated. On the basis of this study, the name *Haemophilus vaginalis* was proposed. Concurrently, Wurch and Lutz (1955) reported the occurrence of the organism in cases of leucorrhea.

Rapidly, a number of reports confirmed the existence of the bacterium and the vaginitis with which it is associated. Ray and Maughan (1956), Gardner, Dampeer, and Dukes (1957), Brewer, Halpern, and Thomas (1957), Edmunds (1959), Heltai (1959), and Gardner and Dukes (1959) reported on both the clinical and bacteriological aspects of *H. vaginalis*. Lutz, Grooten, and Wurch (1956), Pittman, Leopold, and Dukes (1956), Amies and Jones (1957), and Edmunds (1960) reported primarily on the bacteriological and taxonomic aspects of the organism. Throughout these reports, the descriptions of the organism have been essentially in agreement, with various relatively minor inconsistencies. However, none has elucidated fully the properties or nutritional requirements that are essential to establish the proper taxonomic position of this bacterium.

MATERIALS AND METHODS

Cultures. All cultures were isolated in this laboratory from the vaginas of patients with vaginitis in Houston, Texas. The details of diagnosis of *Haemophilus vaginalis* vaginitis and the

technique of isolation have been reported elsewhere (Gardner and Dukes, 1955, 1959; Gardner et al., 1957). All cultures selected for study were isolated and checked for purity by repeated single colony transfers to solid media. The majority of the cultures utilized in this study were preserved by freeze-drying within 30 days after isolation. Recently restored cultures were used for comparative studies. Two isolates were maintained for 250 and 500 transfers, respectively, for determination of stability of growth and metabolic characteristics. Stock cultures were transferred at 48-hr intervals on Casman's blood agar medium (Casman, 1947) containing 5 per cent fresh defibrinated rabbit blood and incubated at 36 to 37 C in a candle-jar.

Media. Casman's blood agar and the Casman formula without agar were utilized as basic solid and liquid media. Fresh defibrinated rabbit blood (5 per cent) or rabbit serum (10 per cent) and yeast extract (5 per cent) were added as indicated. The yeast extract was prepared by the method of Cohen and Wheeler (1946) as modified by Amies and Jones (1957). Cystine trypticase agar medium (BBL), containing 10 per cent rabbit serum, 5 per cent yeast extract, and 1.0 per cent of the carbohydrate, were used for fermentation tests. Media employed for growth factor determinations will be described in that section.

RESULTS

Morphology. Stained bacteria from 18-hr cultures of ten strains were measured. The bacteria appeared as small, discrete rods with rounded ends measuring 0.3 to 0.6 by 1.0 to 2.0 μ . Most strains demonstrated a moderate degree of pleomorphism, occasionally appearing in end-to-end pairs or in small palisading clumps. When stained by the Hucker modification of the Gram method (figure 1), the cells were consistently gram-negative in young cultures (6 to 36 hr). Older cultures (72 to 96 hr) displayed tendencies toward pleomorphism and uneven

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Figure 1. Gram stain of film of 18-hr culture of *Haemophilus vaginalis*.

Figure 2. Giemsa stain of film of 18-hr culture of *H. vaginalis*.

Figure 3. Colonies of *H. vaginalis* on Casman's blood agar after 24 hr incubation at 37 C, showing glossy, "dew-drop" appearance.

Figure 4. Colonies of *H. vaginalis* on Casman's blood agar after 60 hr incubation at 37 C, showing dull, granular appearance.

Figure 5. Growth of *H. vaginalis* in thioglycolate broth with 1 per cent serum and 1 per cent yeast extract after 24 hr incubation at 37 C.

Figure 6. Growth of *H. vaginalis* in Casman's broth with 5 per cent rabbit blood after 24 hr incubation at 37 C.

staining. The uneven staining was associated with the formation of involution forms containing poorly defined granules which tended toward gram-positivity. When stained by the Giemsa method (figure 2), the bacteria showed both uniform and bipolar staining. *H. vaginalis* does not form endospores and capsules have not been demonstrated by the india ink, Anthony, Leifson, or Hiss methods of capsule determination.

Growth characteristics. (1) Solid agar:—On Casman's rabbit blood agar after 24 hr incubation at 36 to 37 C (figure 3), the colonies appear as minute, convex, circular, entire, homogeneous, smooth, translucent, colorless, "dew-drop" colonies measuring 0.1 to 0.8 mm in diameter. Upon further incubation, the colony surface becomes dull and the internal structure finely granular (figure 4). Iridescence is not apparent. The majority of strains produce a green to brown discolorative type of hemolysis on Casman's medium. Smooth-rough variation has not been observed; the colonial morphology has remained constant even after repeated transfers. Visible growth has appeared on solid media after 12 to 16 hr of incubation at 37 C, and the organism remains viable for only 48 to 72 hr at that temperature. Viability was maintained for 2 to 3 days when cultures were refrigerated at 3 C following 18 hr incubation at 37 C in a candle-jar. Length of viability at room temperature (20 to 24 C) ranged from 3 to 5 days.

(2) Semifluid media:—In thioglycolate broth (BBL) containing 0.07 per cent agar, 1.0 per cent serum and 1.0 per cent yeast extract, *H. vaginalis* produced small, 1 to 2 mm, discrete, puff-ball aggregations dispersed throughout the upper 75 per cent of the fluid column (figure 5). Thioglycolate broth was found to be incapable of supporting growth without the addition of serum and yeast extract. In cystine trypticase agar medium, containing 0.35 per cent agar, 10 per cent serum, and 5 per cent yeast extract, the growth was somewhat granular, following the track of the inoculating loop, with maximal growth appearing in the upper portion of the column. In both the thioglycolate broth and the cystine trypticase agar medium, viability persisted for 5 to 6 days at 37 C.

(3) Broth:—In Casman's medium with 5 per cent rabbit blood, from which the agar had been omitted, *H. vaginalis* produced a turbid, diffuse

growth appearing in 12 to 16 hr (figure 6). Such cultures have remained viable at 37 C for 60 to 72 hr.

(4) Temperature:—All of the ten strains tested multiplied at temperatures between 28 and 42 C (water bath and incubator) and remained viable for at least 48 hr. Optimal temperature was 36 to 37 C. None of the strains multiplied at temperatures above 45 C and all failed to survive after incubation at 56 C for 20 min.

(5) Oxygen requirements:—All of the 20 strains tested produced luxuriant growth when cultivated on Casman's blood agar at 37 C in a candle-jar. All grew well aerobically, but growth under strict anaerobic conditions varied with the method of establishing anaerobiosis. All 20 strains grew anaerobically in hydrogen whereas no growth was obtained in pyrogallate and phosphorus jars. In Brewer modification, thioglycolate broth with serum and yeast extract, growth occurred in the upper 75 per cent of the fluid column. However, all strains failed to survive serial transfer in this medium.

Nutritional requirements. All of the strains of *H. vaginalis* required blood or serum and yeast extract as determined by serial passage in broth. No observable growth occurred beyond the second passage in the absence of these materials.

Several factors complicated the early portion of this study. Many of the standard formulation media were found to be inhibitory as solid media and to support only sparse growth as liquid media even with the addition of 10 per cent blood. Part of this inhibitory effect was traced to the presence of a water-soluble component of purified agar which could be eliminated by following the formulation of Casman (utilizing specially washed agar such as Noble agar (Difco)). Also, the addition of 0.1 per cent commercial corn starch (Argo) markedly stimulated growth in complex media. Since neither soluble starch nor dextrin, singularly or in combination, produced comparable stimulation of growth, part of the activity of commercial starch was postulated as absorption of toxic or inhibitory material.

With the addition of 0.1 per cent starch, several media were found to support growth of *H. vaginalis* when enriched with 5 per cent blood or 1 per cent serum and 1 per cent yeast extract: Casman's broth, beef heart infusion broth,

trypticase soy broth (BBL). The most luxuriant growth was obtained with Casman's broth and this medium was utilized as the standard medium for maintenance of stock cultures and preparation of seed cultures for experimental procedures.

Efforts to establish definitively the X and V growth factor requirements by the classic methods proved disappointing. The strains grew sparsely, if at all, on either Fildes peptic digest beef heart infusion agar (Fildes, 1920) or Levinthal agar (Levinthal and Fernbach, 1922). The addition of 10 per cent rabbit serum to Fildes agar enhanced growth, but the yield was far below that obtained on Casman's agar. The satellite phenomenon with *Staphylococcus aureus* was not observed.

Since it was thought that highly complex media might contain inhibitory as well as stimulatory materials, a number of relatively less complex nutrient materials were tested for ability to support growth of *H. vaginalis*. Accordingly, peptone, neopeptone, proteose-peptone no. 3, tryptose, casamino acids, and casitone (products of the Difco Laboratories), and gelsate, lactalsate, phytone, trypticase soy broth, (products of the Baltimore Biological Laboratory) were tested at 1.0 per cent concentrations with the addition of 1.0 per cent serum, 1.0 per cent yeast extract, and 0.1 per cent starch. Of these only the trypticase soy broth and casitone (pancreatic digest of casein) produced growth that could be maintained through serial passage. Since the casitone constituted the less complex nutrient of the two preparations, it was utilized for further growth factor studies.

A basic minimal medium consisting of casitone (0.5 per cent), starch (0.1 per cent), and rabbit serum (1.0 per cent) which supported sparse growth was formulated. When various nutrients were incorporated into this basal medium, the stimulatory or inert activity was compared by turbidimetric measurement of resulting growth of the organism. Related amino acids were tested initially in seven groups as described by Ajello et al. (1956).

Group A: Glycine, DL- α -alanine, β -alanine, DL-serine, DL-threonine.

Group B: DL-Valine, L-leucine, DL-isoleucine.

Group C: L-Proline, L-tryptophan, L-tyrosine, DL-phenylalanine.

Group D: L-Lysine, L-arginine, L-histidine, DL-ornithine.

Group E: Cysteine hydrochloride, DL-methionine, L-cystine.

Group F: L-Aspartic acid, L-asparagine, L-glutamic acid, L-glutamine.

Group G: Hydroxy-L-proline.

All seven groups were tested separately and in various combinations (table 1). All of the above compounds were tested at a concentration of 5 mg per ml of medium.

Only group F or combinations of groups containing group F exhibited growth-promoting properties. Group E, when added to group F, gave an additive effect. Consequently, these two groups were subdivided and tested. L-Aspartic acid and L-glutamic acid, both singularly and in combination, displayed active growth-promoting properties. Of the remaining compounds in groups E and F, only cysteine hydrochloride showed activity and this was evident only when added to either or both L-aspartic and L-glutamic acids.

Cozymase (co-dehydrogenase 1, diphosphopyridine nucleotide, V factor) (0.5 mg per ml of medium) and hematin (0.2 mg per ml of medium) produced additional growth-promoting effects when added to the basal medium containing aspartic and glutamic acids, although neither exhibited any effect in the absence of these amino acids. The combination of cozymase, hematin, aspartic acid, and glutamic acid provided the maximal growth-promoting effect in this series. However, none of the combinations studied approached the growth-promoting effect achieved by the combination of 1.0 per cent serum and 1.0 per cent yeast extract. Thus, it appears that some major factor other than the classic X and V factors remains undetermined.

To evaluate further the importance of glutamic and aspartic acids in relation to the X and V factors, the concentration of serum in the basal medium was reduced to 0.1 per cent. At this concentration, the basal medium did not support the growth of *H. vaginalis* and the addition of glutamic and aspartic acids was without effect. The addition of cozymase to the amino acid-basal medium mixture promoted measurable growth (table 1). Hematin and cysteine also exhibited growth-promoting activity, both singularly and when combined individually with cozymase.

Attempts to utilize the procedures of Herbst and Snell (1949) to elaborate the unknown factors by the use of chemically defined media

TABLE 1
Growth of Haemophilus vaginalis in basal media with the addition of amino acids^a

Basal Media	Turbidity ^b										
	Groups of amino acids ^c							Mixtures of groups of amino acids			
	A	B	C	D	E	F	G	AB CD G	EF	AD EF	ABC DEF
With 1.0 per cent rabbit serum.....	96	96	96	96	96	81	97	97	72	74	83
With 0.1 per cent rabbit serum.....	98	98	98	98	98	98	99	99	95	95	99
With 0.1 per cent rabbit serum and cozymase ^d	98	98	98	98	98	85	98	99	74	76	87
With 0.1 per cent rabbit serum, cozymase, and hematin ^d	97	97	97	97	97	78	97	99	68	70	79

^a All amino acids tested at concentrations of 5 mg per ml of medium. Basal medium consisted of casitone (0.5 per cent) and corn starch (0.1 per cent).

^b Per cent of incident light transmitted; uninoculated medium = 100.

^c The components of each group are: Group A: Glycine, DL- α -alanine, β -alanine, DL-serine, DL-threonine. Group B: DL-Valine, L-leucine, DL-isoleucine. Group C: L-Proline, L-tryptophan, L-tyrosine, DL-phenylalanine. Group D: L-Lysine, L-arginine, L-histidine, DL-ornithine. Group E: Cysteine hydrochloride, DL-methionine, L-cystine. Group F: L-Aspartic acid, L-asparagine, L-glutamic acid, L-glutamine. Group G: Hydroxy-L-proline.

^d Cozymase (diphosphopyridine nucleotide), 0.5 mg per ml of medium; hematin, 0.2 mg per ml of medium.

were unsuccessful. No growth was obtained in any of the combinations of amino acids, vitamins, minerals, etc., tested with or without the addition of serum or yeast extract, singularly or in combination.

Utilization of paper-strip and continuous-flow curtain electrophoresis for physical separation of serum components failed to elucidate the nature of the growth-promoting activity of serum. Comparable fractionation of the yeast extract has not been attempted.

Biochemical reactions. *H. vaginalis* is oxidase negative and is not soluble in sodium deoxycholate. Indole is not produced. Reduction of nitrates to nitrites is variable in speed and intensity among the strains tested. Some strains reduced nitrates rapidly and gave strong reactions whereas others were slow and weak in producing detectable nitrite.

(1) Carbohydrate fermentation:—Thirty-five strains were tested and found to produce acid from glucose, maltose, dextrin, starch, and glycogen within 48 hr. Acid was produced slowly (4 to 7 days) and weakly from arabinose, rhamnose, levulose, and xylose. There was no action in lactose, galactose, sucrose, mannitol, trehalose,

glycerol, raffinose, salicin, inositol, inulin, sorbitol, or dulcitol. The fermentative reactions were not strongly acidic, the lowest reading attained was pH 6.2.

(2) Hemolysin production:—The majority of strains produced a green to brown discoloration on Casman's rabbit blood agar. The edge of the zone of discoloration around the colonies was indistinct, and upon microscopic examination, many of the erythrocytes at the edge of the zone were noted to be intact. Production of a true filterable hemolysin in Casman's broth active against human, sheep, or rabbit erythrocytes was not demonstrated.

Pathogenicity. Efforts to infect experimentally mice, guinea pigs, rats, and rabbits with *H. vaginalis* by the intravenous, intracutaneous, subcutaneous, and intraperitoneal routes were unsuccessful. Both freshly isolated and stock strains were employed in dosages ranging from 10 to 1,000 million organisms. All cells utilized for injection were harvested from 18-hr Casman serum-yeast extract broth cultures. The incorporation of 3 per cent gastric mucin in the inoculum for the subcutaneous and intraperitoneal routes of injection failed to influence the experi-

mental results. Pretreatment of the animals with cortisone acetate (25 mg per kg of body weight injected subcutaneously daily for 7 days prior to injection of the living organisms) also failed to induce infection with the inocula and the four routes listed above. Clinical evidence of pathogenicity in humans has been presented elsewhere (Gardner and Dukes, 1955).

Agglutination. Antisera against four strains were induced in rabbits by the repeated intravenous injection of large numbers of organisms. A standard tube agglutination procedure was used for determination of antibody titers (Evans, 1957). Heat-killed (60 C for 30 min) organisms from 18-hr Casman blood agar plate cultures were adjusted in isotonic saline to a density corresponding to a McFarland no. 2 tube. Each antiserum agglutinated its specific strain at relatively high titers (1:1,024) and gave cross-reactions with other strains at lower titers (1:64 to 1:256). Spontaneous flocculation of some laboratory-adapted strains complicated the attempted serological analysis. No cross-reaction with three strains of *H. influenzae*, two strains of *H. parainfluenzae*, and one strain of *H. aegyptius* was noted with any of the four antisera of *H. vaginalis*.

DISCUSSION

The bacteriological descriptions of *H. vaginalis* reported to date are relatively consistent although inadequate. Amies and Jones (1957) state that all strains reduce nitrates to nitrites, whereas Leopold (1953) and Lutz et al. (1956) assert that nitrate reduction does not occur. Two strains supplied by Amies for comparative study reduced nitrates to nitrites rapidly and intensively. Among our strains, nitrate reduction was found to be a characteristic variable in degree and rapidity.

The degree and type of hemolysis produced on blood agar by *H. vaginalis* is reported variously. Leopold (1953) described a clear hemolysis. Lutz et al. (1956) specified a green halo surrounded by a thin zone of clear hemolysis and proposed the illegitimate trinomial *Haemophilus hemolyticus vaginalis*. Amies and Jones did not describe a reaction on blood agar, but stated that a true hemolysin was not demonstrated. Edmunds (1960) reported that all strains were hemolytic on human blood agar when originally isolated but some became nonhemolytic upon subculture. In blood broth inoculated with heavy suspen-

sions of *H. vaginalis*, he was able to demonstrate hemolysis of human and horse erythrocytes but obtained indifferent results with rabbit blood and no hemolysis with ox and sheep blood. The discrepancies between the various reports possibly are due to variations in media and methods employed. On commercial Casman medium base (Difco) with 5 per cent defibrinated rabbit blood, we observed a green to brown discoloration, but we were unable to demonstrate the formation of a true filterable hemolysin active against human, sheep, or rabbit erythrocytes.

The nutritional studies indicate that the X and V factors are stimulatory accessory growth factors for *H. vaginalis* in the presence of partially digested protein, rabbit serum, and certain amino acids. Under minimal conditions of nutrition, growth was not obtained in the absence of the X and V factors. These observations are amenable to the taxon criteria for the genus *Haemophilus* as described in the seventh edition of *Bergey's Manual of Determinative Bacteriology* (Breed, Murray, and Smith, 1957) and by Wilson and Miles (1955). Since growth of *H. vaginalis* was not obtained serially in the absence of serum, the exact relationship of the X and V factors to the growth of this organism was not determined.

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SUMMARY

Haemophilus vaginalis is a minute, rod-shaped, gram-negative bacillus, which is nonmotile and does not form endospores. Capsules have not been demonstrated. The organism is a strict parasite, growing only in the presence of serum and other accessory growth factors. The X and V factors are stimulatory when the organism is grown in media containing suboptimal concentrations of serum and certain specific amino acids and, under certain conditions, growth does not occur in the absence of the V factor. *Haemophilus vaginalis* appears to be a distinct species that can be separated from the other members of the genus *Haemophilus* serologically and, to a certain extent, by growth and nutritional characteristics and by biochemical activities.

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