

Determination of the Hemin Requirement of *Haemophilus ducreyi*: Evaluation of the Porphyrin Test and Media Used in the Satellite Growth Test

GREGORY W. HAMMOND,† CHANG-JOO LIAN,¹ JOHN C. WILT,¹ WILLIAM L. ALBRITTON,¹
AND ALLAN R. RONALD^{1,3*}

Departments of Medical Microbiology¹ and Medicine,² The University of Manitoba, and Infectious Diseases, Health Sciences Centre,³ Winnipeg, Manitoba R3E 0Z3 Canada

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Gonococcal (GC) agar supplemented with glucose and glutamine was found to be superior to Eugonagar and Trypticase soy agar in demonstrating the hemin requirement of 23 strains of *Haemophilus ducreyi* by the satellite growth test. The porphyrin test confirmed the requirement for exogenous hemein. With the agar dilution technique, using supplemented GC agar, the hemin concentration required to initiate growth was 10 µg/ml, and the optimal hemin concentration to produce growth equivalent to that on chocolate agar was between 200 and 500 µg/ml. On GC agar with added glucose and glutamine, the lowest hemin concentration impregnated in paper disks able to initiate satellite growth was 50 µg/ml. The hemin requirements of these *H. ducreyi* were much higher than that reported for other *Haemophilus* species.

Haemophilus ducreyi is a poorly characterized organism that causes the rare venereal disease, chancroid. By definition, the *Haemophilus* genus requires X factor (hemin or certain other porphyrins) or V factor (nicotinamide adenine dinucleotide [NAD]) or both for growth (13). Many early reports have stressed the difficulties of cultivating this organism, as well as its requirement for whole blood in the medium (1, 2, 10, 12). Lwoff and Pirosky (8) demonstrated the need of X factor for growth of *H. ducreyi*, but later work by Sanderson and Greenblatt (11), Beeson (2), and Ajello et al. (1) suggested that this factor was not required. More recently, Kilian (6) found that two isolates compatible with *H. ducreyi* could not synthesize their own hemin from δ-aminolaevulinic acid (δ-ALA) and required exogenous X factor. Also, their guanine-plus-cytosine content ratios were in the range of the hemin-requiring *Haemophilus* species.

Against this background of controversy, we wish to evaluate the hemin requirement of 19 recent isolates of *H. ducreyi* from a local outbreak of chancroid and four reference strains.

MATERIALS AND METHODS

Bacterial strains. Nineteen strains of *H. ducreyi* were isolated from patients with chancroid between July 1975 and February 1977 at the Health Sciences

Centre, Winnipeg. Four of these strains were obtained in pure culture from inguinal buboes and the remainder from genital lesions of other chancroid patients, including one isolated by A. Manier of the Cadham Provincial Laboratory. Four reference strains of *H. ducreyi* (Collection de l'Institut Pasteur A75, A76, A77, and 54.2) were obtained from the Pasteur Institute in Paris.

Strains were stored at -70°C in skim milk containing 10% glycerol. Organisms, when needed, were subcultured onto chocolate agar (CA) composed of gonococcal medium base (GC) (Difco Laboratories) enriched with 1% hemoglobin powder (Difco) and 1% IsoVitalX (Baltimore Biological Laboratory).

All 23 strains of *H. ducreyi* had the same characteristics as the two CIP isolates characterized by Kilian (6), except that one local and three reference strains did not reduce nitrate. Growth was enhanced in the presence of 5% CO₂ and high humidity.

H. influenzae and *H. parainfluenzae* were obtained from the Department of Clinical Microbiology, Health Sciences Centre, and were used as controls. Colonies from a 48-h culture of *H. ducreyi* and 24-h control cultures were scraped off the CA, suspended in sterile, physiological saline without bacteriostatic preservative, and adjusted to a turbidity corresponding to a McFarland no. 2 nephelometer standard. The inoculum size of *H. ducreyi* was an approximate value because quantitation was difficult due to autoagglutinating and chaining characteristics of this organism. Bacterial suspensions of this inoculum size were used in the study of satellite growth and the effect of hemin concentration.

Synthesis of porphyrins and porphobilinogen. The test fluid used in the synthesis of porphyrins and porphobilinogen contained 2 mM δ-ALA (Sigma

† Present address: Department of Respiratory Virology, Virology Division, Bureau of Laboratories, Center for Disease Control, Atlanta, GA 30333.

Chemical Co.) and 0.8 mM MgSO₄ in 0.1 M phosphate buffer (Sorensen), pH 6.9. A heavy suspension of organisms was made in 0.5 ml of the test fluid using 48-h growth cultures of *H. ducreyi* and 24-h growth of *H. influenzae* and *H. parainfluenzae*. The reaction mixtures were incubated at 37°C for 18 to 24 h. A suspension of the bacterial cells in the test medium without δ -ALA acted as a control.

The production of porphyrins was detected by emission of red fluorescence on exposure of the reaction mixtures to a Wood ultraviolet light lamp against a white background in a dark room. A positive test (red fluorescence) indicated a lack of requirement for exogenous X factor (5).

Porphobilinogen was detected by addition of an equal volume of Kovacs reagent to the previously described mixture of δ -ALA and bacteria. After shaking and standing for a few minutes, a positive reaction was determined by the production of a red color in the lower water phase (7).

Routine satellite growth test for X factor requirement: (i) comparison of three different types of agar media. Bacterial suspensions were streaked in three directions with a cotton swab on separate plates of Trypticase soy agar (TSA) (BBL), Eugonagar (Difco), and GC agar base (Difco). Strips of hemin (4,000 μ g), NAD (100 μ g), and hemin with NAD (2,000 μ g of hemin and 100 μ g of NAD) (BBL Taxo strips—personal communication, BioQuest) were placed aseptically on the agar surface. Plates were incubated at 33°C in a Hotpack CO₂ incubator containing 5% CO₂ and saturated water vapor. Growth around the strips was examined after 24 and 48 h of incubation against a bright background light.

(ii) Comparison of three different types of agar media and nutritional supplements added. Nutritional supplements similar to that of IsoVitaleX (BBL) without NAD (coenzyme I) were added to sterile, cooled TSA, Eugonagar, and GC agar base. The supplemented media contained glucose (1,000 μ g/ml), thiamine · HCl (0.03 μ g/ml), ferric nitrate (0.2 μ g/ml), L-cysteine (259 μ g/ml), guanine (0.3 μ g/ml), adenine (10 μ g/ml), L-glutamine (100 μ g/ml), L-cystine (11 μ g/ml), vitamin B₁₂ (0.1 μ g/ml), cocarboxylase (1 μ g/ml), and *p*-aminobenzoic acid (0.1 μ g/ml). Each organism was streaked, as before, and X, V, and XV strips were added to the agar surface aseptically. Growth around the strips was examined after 24 and 48 h of incubation.

(iii) GC medium supplemented with glucose and glutamine. Filtered, sterilized glucose (0.1%) and glutamine (0.01%) were added to sterile, cooled GC agar base. Satellite growth tests of the organisms for X, V, and XV requirements were performed as before.

Concentration effect of hemin. To determine the concentration of hemin necessary to initiate and produce optimal growth of *H. ducreyi*, two experiments were carried out using a graded series of hemin concentrations.

(i) Agar dilution. Hemin (H-2250 type I bovine, about 93% pure, personal communication—Sigma) dissolved in 1 N NaOH was filtered, sterilized, and added to sterile, cooled GC agar base supplemented with glucose (0.1%) and glutamine (0.01%) to give final concentrations of 0, 1, 5, 10, 20, 50, 100, 200, 500, 750,

and 1,000 μ g/ml. Saline suspensions of 48-h cultures of *H. ducreyi* were inoculated on the surface of the agar, using a Steers-Foltz replicator, starting with the plate of the lowest hemin concentration. CA, as a control, was the final plate inoculated.

The optimal hemin requirement for the bacteria was determined by the presence of growth on the agar surface at the lowest hemin concentration that most closely resembled growth on CA.

(ii) Disk technique. This method utilized the same principle as the satellite growth test of BBL Taxo strips. Sterile paper disks, 12.7 mm in diameter (#740-E, Carl Schleicher and Schuell Co., High Quality Analytical Filter Papers, Keene, N.H.), were impregnated with the same series of hemin concentrations as mentioned earlier in the preceding agar dilution technique. Dried paper disks containing the hemin were used a day after preparation or stored at 4°C and used within a week.

A bacterial suspension was streaked in three directions with a sterile cotton swab to cover the whole surface of NAD-free GC agar supplemented with glucose and glutamine. Hemin-containing paper disks were placed aseptically on the agar surface with a sterile forceps. The plates were incubated at 33°C in 5% CO₂ for 48 h. The presence of growth around the disks was assessed against a bright background light.

RESULTS

All 23 isolates of *H. ducreyi* failed to synthesize porphyrins or porphobilinogen from δ -ALA, confirming a need for an exogenous source of hemin.

H. ducreyi could not be shown to require X factor for growth in routine laboratory techniques using satellite growth test on TSA and Eugonagar (Table 1). GC agar alone could support growth of only two of the isolates in the presence of X factor. With extra nutritional supplements added to TSA, Eugonagar, and GC agar, more of the isolates could be shown to require X factor for growth. However, TSA and Eugonagar could not support the growth of all the isolates of *H. ducreyi* even with added supplements. All 23 isolates of *H. ducreyi*, plated on NAD-free GC agar with all 11 nutritional supplements, required X factor for growth. In preliminary work we found that glucose and L-glutamine were the two essential components of the IsoVitaleX that would support growth of *H. ducreyi* in the presence of X factor on GC agar. Table 1 shows that GC agar with these two factors could support growth of *H. ducreyi* around the X strip and was comparable to GC agar with 11 nutritional supplements.

Table 2 shows the amount of hemin required for the growth of *H. ducreyi*. By the agar dilution technique, *H. ducreyi* could grow at as low a hemin concentration as 10 μ g/ml. However, only 21% of the isolates could grow at this level, and their growth was minimal and hazy. Easily visi-

TABLE 1. *Effect of media on satellite growth of 23 isolates of H. ducreyi around the X factor*

Test for satellite growth ^a	Medium						
	TSA	Eugonagar	GC agar	TSA + ISV ^b without NAD	Eugonagar + ISV without NAD	GC agar + ISV without NAD	GC agar + glucose + glutamine
X strip	0	0	2 (9) ^c	8 (35)	9 (39)	23 (100)	23 (100)
V strip	0	0	0	0	0	0	0
XV strip	0	0	2 (9)	9 (39)	4 (17)	23 (100)	23 (100)

^a X strip, Hemin (BBL, Taxo strip); V strip, NAD (BBL, Taxo strip); XV strip, hemin and NAD (BBL, Taxo strip).

^b TSA, Trypticase soy agar; GC, Gonococcal base agar; ISV, IsoVitaleX 1% (BBL); NAD, Nicotinamide adenine dinucleotide.

^c Number of isolates that grew; numbers in parentheses indicate percentage.

TABLE 2. *Hemin concentration effect on growth of 23 isolates of H. ducreyi*

Hemin Conc ($\mu\text{g/ml}$)	Technique used				
	Agar dilution				Paper disk
	No growth	Minimal growth (+)	Moderate growth (++)	Growth equivalent to CA ^a (+++)	
0	23 (100) ^b	0	0	0	0
1	23 (100)	0	0	0	0
5	23 (100)	0	0	0	0
10	18 (78)	5 (22)	0	0	0
20	15 (65)	8 (35)	0	0	0
50	3 (13)	4 (17)	16 (70)	0	3 (13)
100	2 (9)	0	21 (91)	0	16 (70)
200	2 (9)	0	0	21 (91)	19 (83)
500	2 (9)	0	1 (4)	20 (87)	22 (96)
750	5 (22)	0	1 (4)	17 (74)	23 (100)
1,000	5 (22)	1 (4)	1 (4)	16 (70)	23 (100)

^a CA with 1% IsoVitaleX (BBL).

^b Number of isolates that grew; numbers in parentheses indicate percentages.

ble growth was expressed by 69% of the strains when the hemin concentration was raised to 50 $\mu\text{g/ml}$. Although over 90% of the isolates grew at 100 μg of hemin per ml, their growth was not comparable to growth on control CA until the hemin concentration was raised to 200 $\mu\text{g/ml}$. The paper disk method showed that between 500 and 700 μg of hemin was required in the disk to enable all 23 isolates of *H. ducreyi* to express their requirement for X factor. The lowest hemin content in the disk that supported growth was 50 $\mu\text{g/ml}$. As hemin content in the paper disks was increased, more isolates of *H. ducreyi* were able to express their requirement of X factor for growth, and the area of growth around the disks was larger.

DISCUSSION

These series of experiments were designed to show the absolute requirement for exogenous hemin by *H. ducreyi*.

Satellite growth around an X strip of *H. ducreyi* could not be accurately evaluated on such commonly used media as TSA and Eugonagar.

Evans and Smith (3) also observed that the choice of medium altered the satellite test for *H. influenzae* and *H. parainfluenzae*. Nutritional supplements besides hemin were important for the growth of *H. ducreyi*. The addition of nutritional supplements (identical to IsoVitaleX except without NAD) to TSA, Eugonagar, and GC agar base increased the number of *H. ducreyi* isolates that demonstrated an X-factor requirement. GC agar base supplemented with only glucose and glutamine was comparable to GC agar base with all 11 nutritional supplements added in its ability to demonstrate the X-factor requirement of all 23 *H. ducreyi* isolates. The use of IsoVitaleX without NAD has shown that growth stimulation of this organism by hemin was independent of NAD. The need for exogenous hemin was confirmed by the negative porphyrin test, which demonstrated the lack of enzymatic capability of this organism to synthesize hemin from δ -ALA.

Beeson (2) found that the addition of X factor alone to his basal medium would not support the growth of this organism, and he postulated

that either X and V factors were not essential, or that other factors in whole blood were needed for *H. ducreyi* growth. The proteose peptone broth basal medium used by him may not have been adequate to show the X-factor requirement of *H. ducreyi*. Ajello et al. (1) used a modification of Pickett and Stewart's method (9) to test for satellite growth of *H. ducreyi* around streaks of catalase-positive and -negative organisms. However, the medium used was similar to unsupplemented TSA and Eugonagar, and, therefore, it is not surprising that Ajello et al. (1) could not establish the X-factor requirements of *H. ducreyi*.

The importance of the hemin concentration in the medium is shown by the need for a level of 200 μg of hemin per ml, as determined by the agar dilution method, before optimal growth was observed. Higher hemin levels appeared to inhibit growth of some strains, an observation which could not be explained. Two isolates failed to grow at all levels of hemin concentration. The disk content required in the satellite test is at least 500 $\mu\text{g}/\text{ml}$ in order to allow growth of all *H. ducreyi* isolates, a level which is achieved in commercially available hemin-impregnated strips. A strain variation in the requirement for different levels of hemin may also exist. The hemin concentration required for optimal growth of this organism is, however, much higher than for other species of *Haemophilus*, such as *H. influenzae*, whose optimal hemin requirement is 2 to 10 $\mu\text{g}/\text{ml}$ (4).

These studies demonstrate the exogenous hemin requirement of *H. ducreyi* and also suggest that other variables such as media and nutritional supplements are important in demonstrating the X-factor requirement of this organism. The concentration of hemin also has to be considered. The high hemin requirement of this organism may account for previously ob-

served negative biochemical reactions in which only 10 μg of hemin per ml was used in the basal medium (6).

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