Clear Broth and Plate Media for Culture of *Haemophilus ducreyi*

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Using catalase as a source of heme, we have developed both clear plate and broth media for the growth of *Haemophilus ducreyi*, the causative agent of chancroid. In the broth medium, the growth phase of the organism can be monitored and the organisms achieve a cell density of $>10^8$ CFU/ml.

Haemophilus ducreyi is the causative agent of chancroid, a sexually transmitted disease characterized by genital ulcers and inguinal lymphadenopathy. In Africa, chancroid is one of the most common sexually transmitted diseases and increases the risk of acquiring human immunodeficiency virus infection (14, 23). In the United States, the incidence of chancroid has been increasing since 1984 and peaked in 1987 (6). *H. ducreyi* is a fastidious organism and, even with complex selective media, is isolated from only 50 to 80% of suspected chancroid cases (16). However, only 14% of sexually transmitted disease clinics in the United States have culture media suitable for isolation of this organism (17). Thus, this disease is undoubtedly underreported, and new methods for growth of this organism are needed. In addition, there is a need for studies on the pathogenesis of chancroid infection; such studies would be facilitated by improved culture media.

H. ducreyi requires heme for growth because it cannot synthesize porphyrins from δ -aminolevulinic acid (7). Thus, culture plates for *H. ducreyi* typically contain hemin, hemoglobin, or whole blood. Since these plates are not clear, hydrolysis of chromogenic reagents incorporated in the media cannot be visualized. In addition, because the organism clumps when it is grown on plates, the preparation of standardized inocula and separation of individual bacteria are difficult. Finally, growth in broth containing hemin, hemoglobin, or whole blood is difficult to monitor because the broth is too turbid to analyze spectrophotometrically. To overcome these problems, we have developed clear broth and plate media for growth of *H. ducreyi* on the basis of previous work indicating that this organism can utilize catalase as a source of heme (3) and requires fetal bovine serum (FBS) for optimal growth (19).

Ten strains of *H. ducreyi* were tested for their ability to grow in both plate and broth media containing catalase (Table 1). All strains were identified as *H. ducreyi* by their typical colony morphology, requirement for hemin but not nicotinamide adenine dinucleotide by using Taxo X and V factor strips (BBL Microbiology Systems, Cockeysville, Md.) on GC agar plates with 1× GGC (100× GGC is 10% glucose, 1% glutamine, 2.6% cysteine) and 10% FBS, and a positive reaction in a taxonomic spot blot test as described for *Campylobacter* spp. (22) but with *H. ducreyi* type strain CIP 542 whole-cell DNA as a probe (data not shown). In this test, test strains of *H. ducreyi* were incubated on chocolate agar plates with fetal bovine

serum (CFS plates) for 24 h and then suspended in L broth. Aliquots (10 μ l) of this suspension were placed on nitrocellulose filters, lysed, fixed, and then hybridized with radiolabelled whole-cell DNA isolated from *H. ducreyi* CIP 542. After excess DNA was removed by washing, the filters were exposed to X-ray film and developed. Darkening of the film comparable to that of the control, *H. ducreyi* CIP 542, identified the test strain as *H. ducreyi*. Other *Haemophilus* spp., including *H. influenzae*, *H. segnis*, *H. parainfluenzae*, and *H. aphrophilus*, served as negative controls in this test.

H. ducreyi strains were routinely cultured on CFS plates which contained GC agar base (Difco Laboratories, Detroit, Mich.), 1% hemoglobin (BBL), 1× GGC, and 10% FBS (GIBCO BRL, Gaithersburg, Md.). The GGC and FBS were added after autoclaving. All plate media were incubated at 35°C in a candle jar.

All *H. ducreyi* strains were stored at -70°C in L broth (10) containing 25% glycerol. To avoid changes in the stock culture that might occur after prolonged laboratory passage, strains were removed weekly from these stock cultures and incubated for 2 days on CFS before inoculation of plate media to be tested. For inoculation of liquid media, growth from these plates was used to inoculate another CFS plate which was incubated for 24 h. A portion of this growth was then removed with a swab and used to inoculate the liquid media to an approximate optical density at 540 nm (OD_{540}) of 0.1.

To test the ability of *H. ducreyi* to grow with catalase as a source of heme, all 10 strains were inoculated onto plates containing GC agar base, 1% XV Factor Enrichment (PML Microbiologicals, Tualatin, Oreg.), 10% FBS, and either 0, 10, 100, 200, 500, or 1,000 μg of catalase (Sigma Chemical Co., St. Louis, Mo.) per ml. XV Factor Enrichment is similar to IsoVitalX (BBL) and contains the following (per liter): 25.9 g of L-cysteine HCl, 1.1 g of L-cystine, 0.03 g of guanine HCl, 0.01 g of vitamin B₁₂, 0.10 g of cocarboxylase, 0.25 g of nicotinamide adenine dinucleotide, 10 g of L-glutamine, 0.02 g of ferric nitrate, 100 g of glucose, 3 mg of thiamine HCl, 13 g of *para*-aminobenzoic acid, and 5 ml of HCl (12 N). For all 10 strains, optimal growth on these media was achieved by using 200 μg of catalase per ml; slightly smaller colonies were observed with 100 μg of catalase per ml, and clearly smaller colonies were present on plates with 10 μg of catalase per ml. Incorporation of 500 and 1,000 μg of catalase per ml in these plates did not increase the colony size. In addition, growth on plates containing 200 μg of catalase per ml (CAT plates) was comparable in size to or only slightly smaller ($<25\%$) than colonies appearing on similar plates containing 1% hemoglobin instead of catalase. Colonies of *H. ducreyi* LA228R on CAT

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TABLE 1. *H. ducreyi* strains used in this study, their relevant features, source, and references

Strain	Relevant feature	Source or reference
LA228R	Derivative of LA228	This study
35000	Clinical isolate, Winnipeg, Canada	W. Albritton ^b (8)
CF101	Clinical isolate, Seattle, Wash.	HMC stock collection ^c
V-1168	Clinical isolate, Seattle, Wash.	HMC stock collection ^c (21)
CH2	Clinical isolate, Thailand	L. Slaney and A. Ronald ^d
VI49/91	Clinical isolate, Rwanda	P. Piot ^e
V-180	Clinical isolate, Rwanda	P. Piot
CIP 542	Type strain	S. Morse ^f (8)
CIP A75	Reference strain	L. Slaney and A. Ronald (8)
CIP A77	Reference strain	L. Slaney and A. Ronald (8)

^a LA228R is a derivative of LA228 isolated from a dermal lesion in the temperature dependent rabbit model (15). LA228 is a clinical isolate obtained from J. R. Greenwood, UCLA, Los Angeles.

^b Provincial Laboratory of Public Health, Edmonton, Alberta, Canada.

^c HMC, Harborview Medical Center, Seattle, Wash.

^d University of Manitoba, Winnipeg, Canada.

^e Institut of Tropical Medicine, Antwerp, Belgium.

^f Centers for Disease Control and Prevention, Atlanta, Ga.

plates (Fig. 1B) and on similar plates containing 1% hemoglobin (Fig. 1A) after 3 days of growth are shown. No growth or pinpoint colonies of strain LA228R were seen in the medium lacking catalase and hemoglobin (Fig. 1C), confirming that catalase or other sources of heme are required for growth on these plates.

Clear broth for growth of *H. ducreyi* (Hd broth) was also developed and contained GCB (GC agar base without the agar), 1% XV Factor Enrichment, 100 μ g of catalase per ml, and 10% FBS. When this broth was inoculated and incubated at 35°C with shaking at 200 rpm on a test tube rack placed at an angle of 45° on an Orbital shaker (Bellco Glass, Inc., Vineland, N.J.), optimal growth of strains LA228R, 35000, and CF101 was achieved in 5- to 10-ml volumes in glass culture tubes (13 by 150 mm). Growth in these conditions was much greater than with 2 ml, presumably because the concentration

of oxygen in this broth was optimal for growth of *H. ducreyi*, which is microaerophilic. The OD₅₄₀ achieved after 24 h of growth in Hd broth was 0.9 to 2.0 for all strains tested. Growth of three strains, LA228, 35000, and CF101, in Hd broth is plotted in Fig. 2A. In this medium, these strains had doubling times of 3 to 4 h.

The viability of *H. ducreyi* grown in Hd broth was also studied by plating 100- μ l samples onto CFS plates. As shown in Fig. 3, the number of viable cells of LA228R and 35000 increased relative to the OD₅₄₀, reaching densities of 8×10^7 and 2.5×10^8 CFU/ml at 24 h, respectively. At 48 h, viability decreased, with only 8×10^3 and 5×10^6 CFU of LA228R and 35000 per ml (data not shown). Similar growth after 24 h was achieved with strain CF101, which reached a cell density of 10^8 CFU/ml and an OD₅₄₀ of 1.0.

The appearance of all 10 *H. ducreyi* strains after 24 h of growth in Hd broth was also observed. Strains were harvested from the broth or plates into 1.7-ml microcentrifuge tubes, centrifuged for 1 min (Microfuge II; Beckman Instrument Inc., Palo Alto, Calif.), suspended in distilled water, and vortexed before staining with crystal violet. All strains except CIP A75 and CIP A77 appeared as single chains and occasionally aligned as parallel chains; the latter two strains appeared as single organisms. The chaining of LA228R and 35000, compared with the lack of chains seen with CIP A75, after 24 h of growth in Hd broth is shown in Fig. 4. This can be compared with the single bacteria and clumps seen with all 10 strains

FIG. 1. Colonies of *H. ducreyi* LA228R on plates containing GC agar base, 1% XV supplement, 10% FBS, and either 1% hemoglobin (A) or 200 μ g of catalase per ml (B). No growth of this strain was seen on plates containing GC agar base, 1% XV supplement, and 10% FBS without hemoglobin or catalase (C).

FIG. 2. Growth of strains 35000, LA228R, and CF101 in Hd broth with 10% (A) or 40% (B) FBS. Growth of all three strains was enhanced in 40% FBS. A 1:25 dilution of the appropriate strain grown overnight in Hd broth was used as the inoculum.

FIG. 3. Growth of strains LA228R and 35000 monitored by both absorbance at OD₅₄₀ and viability of the organism. The number of CFUs recovered from the broth increased relative to the OD₅₄₀, reaching a density of 8×10^7 and 2.5×10^8 CFU/ml for strains LA228R and 35000, respectively. A 1:25 dilution of overnight cultures of *H. ducreyi* in Hd broth was used as the inoculum.

after 24 h of growth on CFS plates. The appearance of LA228R, 35000, and CIP A75 grown on CFS plates is shown in Fig. 4. Clumping on agar plates makes quantitation of bacteria difficult, and methods to reduce clumping have been devised. For instance, Alfa et al. (4) eliminated larger clumps of bacteria by harvesting growth from plates into broth, letting the larger clumps of *H. ducreyi* settle by gravity, and then collecting the single cells or smaller clumps from the top of the tube. Growth in broth is an alternative method of obtaining nonaggregated bacteria suitable for study in other tests.

Other factors that might affect the growth of *H. ducreyi* in Hd broth were tested by using three representative strains (LA228, 35000, and CF101). No growth was observed in Hd broth without FBS. An OD₅₄₀ of <0.4 after 24 h of growth was obtained when newborn calf serum was substituted for FBS, consistent with the observations by Sottnek et al. (19) that newborn calf or bovine serum could not be substituted for FBS in various formulations of chocolate agar. Growth in Hd broth in which the XV Factor Enrichment was replaced with 1× GGC was comparable to growth in Hd broth with 1% XV Factor Enrichment; 2× GGC did not increase the growth yield. Increasing the catalase concentration to 200 or 500 µg/ml did not increase the growth rate or growth yield. In contrast, the growth rate and yield of all three strains in Hd broth were

FIG. 4. Crystal violet stain of *H. ducreyi* LA228R, 35000, and CIP A75 after 24 h of growth in broth and on plates. After growth in Hd broth, LA228R and 35000 appeared in chains; CIP A75 was present as single bacteria (top panels). All strains appeared as single cells and clumps of cells after 24 h of growth on CFS plates (bottom panels).

enhanced by the addition of 40% rather than 10% FBS (Fig. 2). However, because of the expense of FBS and the adequate growth yield in Hd broth with 10% FBS, we used 10% FBS in all subsequent tests.

Variables that affected growth in Hd broth were identified and standardized. We noticed slight differences in the ability of *H. ducreyi* to grow in different batches of catalase and FBS, which were reduced by using only one lot number. Differences in growth of *H. ducreyi* strains in different lots of catalase (C-6665; Sigma Chemical Co.) could be explained by variations in activity and purity between lots (2,000 to 5,000 U/g). However, the preparation of catalase used was much less expensive (\$10.40/g) than other, more standardized Sigma preparations (Ex C-3155, 50,000 U/g, \$328.30/g). Thus, the same lot number of Sigma catalase C-6665 was used in all subsequent batches of Hd broth.

The additives used to obtain optimal growth of *H. ducreyi* are intriguing. Why FBS but not bovine serum enhanced growth is not known. It has been suggested that serum provides not only nutrients but detoxifying compounds that also enhance the growth of *H. ducreyi* (2). On the basis of the observation that the requirement for FBS could be replaced by 0.2% charcoal, plates containing charcoal were developed for the culture of *H. ducreyi* by Lockett et al. (9). We found that growth of all 10 strains listed in Table 1 was comparable on CFS and charcoal plates (GC agar base, 1% GGC, 1% hemoglobin, 0.2% activated charcoal [Sigma]) (data not shown), and thus charcoal plates rather than CFS were used in the latter part of this study for routine culturing of *H. ducreyi*. The ability of charcoal to substitute for FBS in chocolate agar supports the hypothesis that FBS acts as a detoxifying agent.

The utilization of heme from catalase is perhaps more clinically relevant for *H. ducreyi* than the utilization of heme from hemoglobin. Unlike hemoglobin, which is present predominantly in erythrocytes, catalase is present in many cells in the body (1). Thus, the ability of *H. ducreyi* to lyse erythrocytes (13, 20, 20a) may only be an indicator of its ability to lyse cells in infected tissues in order to acquire catalase.

The uniformity in the amount of catalase required for optimal growth of *H. ducreyi* strains (200 µg/ml) contrasts with the interstrain variability in optimal hemin concentrations (200 to 1,000 µg/ml) described by Hammond et al. (7). While the interstrain differences in utilization of hemin and heme from catalase may reflect differences in the basal medium, strains tested, and techniques used between laboratories, they may also reflect the relative ability of *H. ducreyi* strains to utilize these two compounds. Hemin is relatively insoluble and aggregative in aqueous solutions (5), which could complicate its utilization by bacteria. Further studies are needed to assess the biochemical and uptake pathways necessary for *H. ducreyi* to utilize heme from catalase.

The development of clear broth and plates for the growth of *H. ducreyi* should have many applications. The effect of different additives and inhibitors of growth can be easily monitored by analyzing relative growth rates in Hd broth spectrophotometrically and thus should facilitate studies of the physiology and metabolism of *H. ducreyi*. The ability to monitor growth phase in Hd broth should aid in optimal expression of many genes, including potential virulence genes in *H. ducreyi*. CAT plates should also be useful for the development of tests based on colorimetric indicators in the agar. For example, transposon mutagenesis of *H. ducreyi* by using *phoA* fusions could be used to enhance the detection of potential virulence genes; expression of the *phoA* gene product is detected as blue colonies on clear plates (11). Similarly, *lacZ* fusions (18), also detected as

blue colonies on clear plates, could be used to monitor the expression of virulence genes.

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