

## Isolation and Identification of *Haemophilus ducreyi* in a Clinical Study

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Seventeen strains of *Haemophilus ducreyi* were isolated from genital lesions which were negative for syphilis by dark-field examination. Media used for primary isolation at various times during the study were enriched chocolate agar, chocolate agar plus vancomycin (3  $\mu\text{g/ml}$ ), rabbit blood agar plus vancomycin (3  $\mu\text{g/ml}$ ), fetal bovine serum agar, and fetal bovine serum agar plus vancomycin (3  $\mu\text{g/ml}$ ). *H. ducreyi* was isolated on chocolate agar plus vancomycin from 10 of 14 patients found to be positive on one or more media, on rabbit blood agar plus vancomycin from 16 of 17 patients, and on fetal bovine serum agar plus vancomycin from 9 of 11 patients. Sera from six animal species were tested to determine if any would support the growth of *H. ducreyi*. Horse and rabbit sera supported light growth of some strains. Fetal bovine serum supported good growth of all strains included in the study. Biochemical and physiological tests were done on the 17 isolates, a reference strain of *H. ducreyi*, and two reference strains of *Haemophilus haemoglobinophilus*. The results agreed with those reported by Kilian, except that *H. ducreyi* produced alpha-hemolysis in stabs on rabbit blood agar and was oxidase positive, three strains were urease positive, and  $\text{CO}_2$  improved the growth of seven strains. All 17 isolates were  $\beta$ -lactamase positive. The reference strains were  $\beta$ -lactamase negative.

Chancroid is usually diagnosed on the basis of a combination of the clinical picture and exclusion of other etiologies of genital lesions. *Haemophilus ducreyi*, the causative agent of chancroid, is a fastidious organism, and many workers have found it difficult or impossible to isolate in pure culture. Recovery rates of *H. ducreyi* from chancroid-like lesions have been low in most studies. Two reasons for this have been postulated: chancroid-like lesions can be caused by other organisms such as herpes simplex virus and *Treponema pallidum*, and the clot culture technique often used for culturing *H. ducreyi* is not sufficiently sensitive (5).

In 1978, Hammond et al. (8) described a culture technique which appears to be superior to previously described techniques. By using an agar medium containing vancomycin, they were able to isolate *H. ducreyi* from 8 of 16 suspected cases.

In this study, sera from human and five animal sources were evaluated as supplements for media for cultivating *H. ducreyi*. The effectiveness of five isolation media, including the medium used by Hammond et al., was compared; and the characteristics of 17 isolates of *H. ducreyi* were determined and compared with those of a reference strain of *H. ducreyi* and another X-factor-dependent species of *Haemophilus*, *H. haemoglobinophilus*.

### MATERIALS AND METHODS

**Isolates.** Seventeen strains of *H. ducreyi* were isolated in the Special Bacteriology Section at the Center for Disease Control from clinical specimens between 1 August 1978 and 7 February 1979. One reference strain of *H. ducreyi* (A77) from the Pasteur Institute, Paris, France (sent to us by Gregory W. Hammond), and two reference strains of *H. haemoglobinophilus* (*canis*) (NCTC 1659 and NCTC 8540) were also studied.

**Sera for media enrichment.** One lot each of bovine, newborn calf, horse, rabbit, and pooled human sera and seven lots of fetal bovine sera (FBS) were tested. The sera were not heat treated. Plates were prepared with heart infusion agar (HIA) and 10% (vol/vol) serum. Plates consisting of HIA base and 5% defibrinated rabbit blood (RBA) were used as controls. Four strains of *H. ducreyi*, including the reference strain, were used to inoculate each plate. A heavy suspension (approximating the density of a McFarland no. 2 standard) of growth from a 24-h RBA plate was prepared in sterile normal saline. Each plate was inoculated with one loopful of the suspension and streaked to obtain isolated colonies. The plates (and all subculture media plates used in the study) were incubated at 35°C in a candle jar containing a moistened paper towel and examined after incubation for 24 and 48 h and 5 days. "Best" growth was determined by the approximate number of colonies on the plate and the maximum size of well-isolated colonies after 5 days.

**Agar bases tested.** Six different bases to which

10% FBS had been added were tested in a similar manner. The bases tested were HIA, Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.), Columbia agar (BBL), proteose no. 3 agar (Difco Laboratories, Detroit, Mich.), gonococcal (GC) base agar (BBL), and Trypticase soy agar (BBL).

**Media used for primary isolation.** The following media were used during the course of the study: chocolate agar (CA) plates consisting of GC medium base (Difco) enriched with 1% hemoglobin (Difco) and 1% IsoVitaleX (BBL); CA + vancomycin (V), 3 µg/ml; RBA plates + V, 3 µg/ml; plates consisting of HIA and 10% FBS (FBSA); and FBSA plates + V, 3 µg/ml. All of the specimens tested were not cultured on all of the different types of media.

**Source of isolates.** Specimens were obtained from genital lesions which were negative for syphilis by dark-field examination. The patients were examined at sexually transmitted disease clinics in Fulton and DeKalb Counties, Georgia.

**Technique used for primary isolation.** Material for culture was obtained from the base of the ulcer with a cotton-tipped swab moistened with phosphate-buffered saline (pH 7.2) and streaked on approximately one-third of the surface of the plate. The remainder of the plate was streaked with a loop to obtain isolated colonies. The same swab was used to inoculate all of the plates. The order in which each type of medium was inoculated was randomized. The plates were placed in a Brewer jar with a CO<sub>2</sub> GasPak (BBL), which produced an atmosphere of approximately 4% CO<sub>2</sub>, and incubated at 35°C. Colonies suspected of being *H. ducreyi* were transferred to RBA plates, which were incubated in a candle jar at 35°C. These plates were examined at 24, 48, and 72 h. If the colonial morphology seemed typical of *H. ducreyi*, smears were made from the colonies and Gram stained. If the cellular morphology was typical, the isolate was presumed to be *H. ducreyi*, and physiological and biochemical studies were done to confirm identification.

**Biochemical and physiological tests.** The factor requirements of all the strains studied were determined with plates containing (i) GC agar base (GIBCO Diagnostics, Madison, Wis.) supplemented with glucose (0.1%), glutamine (0.01%), and cysteine (0.05%) (as recommended by William L. Albritton, personal communication) and (ii) BBL Taxo *Haemophilus* differentiation strips. Growth from a 24- to 48-h RBA plate was suspended in sterile normal saline at a density approximating that of a McFarland no. 2 standard. The suspension was streaked on the plate, and X, V, and XV factor strips were placed on the surface aseptically and as far apart as possible. A limited number of strains were also tested with GC agar (Difco) supplemented with glucose, glutamine, and cysteine, with HIA, and with HIA supplemented with glucose, glutamine, and cysteine, as bases.

Tests used to detect synthesis of porphyrins and production of catalase, hydrogen sulfide (H<sub>2</sub>S), and alkaline phosphatases were the same as those described by Kilian (9).

Tests and media used to detect oxidase, urease, and indole production and reduction of nitrate to nitrite

are the same as those used routinely in the Special Bacteriology Section laboratory and have been previously described (10). To enhance growth, FBS was added to the broths to produce a concentration of 10% (vol/vol), and 2 drops were added to the surface of Christensen urea agar slants.

All strains were tested for carbohydrate utilization by using Brown's modification of the rapid fermentation test (4) and in heart infusion broths containing 10% FBS, 1% carbohydrate, and 0.004% aqueous phenol red. Selected strains were tested in phenol red broth base (Difco), CTA medium (BBL), oxidation-fermentation medium (Difco), and Mueller-Hinton agar slants with phenol red indicator, each containing 10% FBS and 1% carbohydrate.

Slants of HIA + 10% FBS were used to study temperature and atmospheric conditions. Growth from a 24- to 48-h RBA plate was suspended in heart infusion broth, and 1 drop was placed on the surface of each slant with a capillary pipette. Slants were incubated at 25, 35, and 42°C in a candle jar, at 35°C in air, and at 35°C in a Brewer jar with an anaerobic GasPak (BBL).

Hemolysis of rabbit blood was determined by examining RBA plates which had been inoculated with a loop, stabbed in the area of primary inoculum, and incubated for 48 h.

Cultures were screened for β-lactamase production either by dropping 0.01 ml of the chromogenic cephalosporin nitrocefim (Glaxo, Ltd., Greenford, Middlesex, England) on individual colonies or by suspending a loopful of bacteria in 0.025 ml of nitrocefim in the well of a microtiter plate (11). Individual colonies which turned red within 60 s were considered positive for β-lactamase production. Microtiter wells which developed a red color within 10 min were considered positive.

## RESULTS

Of the various animal sera evaluated as media enrichment to be used for primary isolation, we found that three (bovine, newborn calf, and pooled human sera) did not support the growth of any of the four strains of *H. ducreyi* tested. Two strains grew very lightly on the plates containing horse serum, and colony size varied from punctate to 0.3 mm. Three strains grew very lightly on the rabbit serum plates, and colony size varied from punctate to 0.1 mm. All the strains grew on all the plates containing seven different lots of FBS. Three of the strains produced moderate to heavy growth on all of the plates, and maximum colony size varied from 0.5 to 2 mm. One strain produced light to heavy growth, and maximum colony size varied from punctate to 1.5 mm.

All six agar bases plus FBS that were tested supported the growth of *H. ducreyi* to some extent. Growth was heaviest and colonies were largest on HIA; the next best medium was Mueller-Hinton agar. Strains grew equally well on Columbia agar, proteose no. 3 agar, and GC base

agar and least well on Trypticase soy agar.

Of the five culture media used for primary isolation, the three media containing vancomycin were more satisfactory than the two without vancomycin. Eight of the specimens from which *H. ducreyi* was isolated were inoculated onto all three vancomycin-containing media. Six of the specimens were inoculated onto CA + V and RBA + V only, and three were inoculated onto RBA + V and FBSA + V only. The results are presented in Table 1. *H. ducreyi* was isolated on CA + V from 10 of 14 patients found to be positive on one or more media, on RBA + V from 16 of 17 patients, and on FBSA + V from 9 of 11 patients. We isolated *H. ducreyi* on RBA + V from four patients who had negative cultures on CA + V plates and from one patient whose culture was negative on FBSA + V. One patient had a positive culture only on FBSA + V, and two strains grew much better on FBSA than on RBA. The plates without vancomycin usually were overgrown with a mixed flora of organisms. We did, however, isolate *H. ducreyi* in almost pure culture on three of these plates cultured from lesions containing large numbers of organisms.

Pure cultures of *H. ducreyi* have a distinctive colonial morphology. After 24 h of incubation, growth is light to moderate and may look "heaped up" in the heavy areas. Colonies vary in size from punctate to 0.5 mm in diameter. On RBA and CA plates the colonies vary from translucent to semiopaque. After 48 h the colonies are all semiopaque. On FBSA plates the colonies are slightly mottled and iridescent when viewed through a dissecting microscope. On all three types of media the growth is difficult to remove with an inoculating needle, and as previously

noted by several workers (6, 8), the colonies can be pushed intact across the surface of the plate. The overall appearance may be that of a mixed culture. We noted that subcultures of the isolates grew much better on all three media if the plates were no more than 2 weeks old and appeared moist on the surface.

On primary isolation from clinical material, if a number of other organisms were growing along with *H. ducreyi*, the colonies of *H. ducreyi* were usually punctate and uniform in size after 24 to 48 h and difficult to distinguish from other fastidious organisms such as some *Corynebacterium* sp., *Haemophilus parainfluenzae*, and *Haemophilus vaginalis*. In fact, six of our isolates of *H. ducreyi* thought to be pure cultures were later found to be mixed with *H. vaginalis*.

The microscopic morphology of *H. ducreyi* organisms grown in mixed culture on a solid medium is less distinctive than that of organisms grown in pure culture (3). There are some chains composed of coccoid forms and rods of varying lengths, but the dramatic characteristic of 5 to 20 or more chains in parallel alignment is seldom seen. We found that identification of *H. ducreyi* could not be based on cellular morphology observed on Gram-stained smears made from primary isolation plates because some strains of *H. parainfluenzae* isolated during this study had similar morphologies. Further characterization was necessary to establish the identification.

The results obtained from our biochemical tests and physiological studies are listed in Table 2. All 17 of our isolates, the reference strain of *H. ducreyi*, and the 2 reference strains of *H. haemoglobinophilus* grew around the X and XV Taxo strips when tested on supplemented GIBCO GC agar base. When supplemented Difco GC agar base and supplemented HIA base were used, several of our isolates did not grow around either of the strips. None of the strains tested was able to synthesize porphyrins.

All of the Special Bacteriology Section isolates were  $\beta$ -lactamase positive. The three reference strains were  $\beta$ -lactamase negative.

All of the strains produced alpha-hemolysis in the stabs after 48 h of growth on RBA plates, were oxidase positive, and were able to grow under anaerobic conditions at 35°C. An atmosphere containing increased CO<sub>2</sub> improved the growth of seven of our isolates. All but one of the *H. ducreyi* strains and one strain of *H. haemoglobinophilus* grew at 25°C. Except for one of the *H. haemoglobinophilus* strains, which grew well, the strains tested grew very lightly or not at all at 42°C.

The two strains of *H. haemoglobinophilus* produced H<sub>2</sub>S and were catalase and indole pos-

TABLE 1. Isolation of *H. ducreyi* on media containing vancomycin (V)

Media used to culture specimens <sup>a</sup>	No. of positive specimens tested <sup>b</sup>	No. of specimens positive on:		
		CA + V	RBA + V	FBSA + V
CA + V, RBA + V, FBSA + V	8	6	7	6
CA + V, RBA + V	6	4	6	NT <sup>c</sup>
RBA + V, FBSA + V	3	NT	3	3
No. positive/no. tested		10/14	16/17	9/11

<sup>a</sup> See text for abbreviations.

<sup>b</sup> Positive specimen = specimen from which *H. ducreyi* was isolated on one or more media.

<sup>c</sup> NT, Not tested.

TABLE 2. Characteristics of patient isolates of *H. ducreyi*, a reference strain of *H. ducreyi*, and reference strains of *H. haemoglobinophilus*<sup>a</sup>

Characteristic	<i>H. ducreyi</i> l'Institut Pasteur A77	Patient isolates (n = 17)	<i>H. hae- moglobi- nophilus</i> NCTC 1659	<i>H. hae- moglobi- nophilus</i> NCTC 8540
X-factor re- quirement <sup>b</sup>	+	+	+	+
V-factor re- quirement <sup>b</sup>	-	-	-	-
ALA, porphy- rins	-	-	-	-
H <sub>2</sub> S (lead ace- tate)	-	-	+	+
Catalase	-	-	+	+
Oxidase	+	+	+	+
Indole	-	-	+	+
Nitrate reduc- tion	-	+	+	+
Urease	-	3(w)	-	-
Alpha hemolytic in RBA "stab"	+	+	+	+
Alkaline phos- phatase	+	+	-	-
β-Lactamase	-	+	-	-
CO <sub>2</sub> improves growth (can- dle jar)	-	7	-	-
Anaerobic growth	+	+	+	+

<sup>a</sup> + indicates all strains positive; - indicates all strains negative. When reactions varied, number indicates the number of positive strains. ALA, Delta-aminolevulinic acid; w, weak.

<sup>b</sup> X-factor, Hemin; V-factor, nicotinamide adenine dinucleotide.

itive and alkaline phosphatase negative. All of the *H. ducreyi* strains were H<sub>2</sub>S, catalase, and indole negative and alkaline phosphatase positive. All of the strains tested except the reference strain of *H. ducreyi* reduced nitrates to nitrites. Three of our isolates were urease positive after 3 to 7 days of incubation. All other strains tested were negative.

Both strains of *H. haemoglobinophilus* utilized glucose, xylose, mannitol, sucrose, and maltose, but not lactose. We are uncertain about the ability of *H. ducreyi* to utilize carbohydrates. Some of the strains produced an apparent weak acid reaction in glucose or glucose and maltose when tested by the rapid fermentation method and also in heart infusion broth, but the reactions obtained could not be consistently repeated.

## DISCUSSION

We found that all three types of culture media used in this study will support good growth of *H. ducreyi*, and when vancomycin is added to the media, all are suitable for use in primary

isolation. Although we found the isolation rate of *H. ducreyi* to be higher on RBA and FBSA than on CA, many laboratories may prefer to use CA because they find the ingredients are more readily available. One advantage of using FBSA for primary isolation is that it does not support the growth of *H. influenzae* or *H. parainfluenzae*, both of which can be mistaken for *H. ducreyi* in mixed cultures on CA or RBA plates.

Several investigators have reported that a moist atmosphere enhances the growth of *H. ducreyi* (2, 3, 7, 11). Our findings agree with theirs. Best growth is obtained when the plates used are no more than 2 weeks old.

Several workers have used rabbit serum as a medium enrichment (1, 3, 5). Ajello et al. (1) found that although rabbit and human sera were best, bovine and porcine sera would also support the growth of *H. ducreyi*. The concentration of serum used by these workers varied from 0.2 to 50%. We found that in the concentration we used (10%), rabbit serum supported the growth of some strains, but only fetal bovine serum supported good growth of all the strains tested.

A number of biochemical and physiological studies can be done on *H. ducreyi* and *H. haemoglobinophilus* if 10% FBS is added to the media to support the growth of the organisms. Although both organisms require hemin (X factor) for growth, *H. ducreyi* can be distinguished from *H. haemoglobinophilus* by its distinctive colonial and cellular morphology, its failure to produce H<sub>2</sub>S, catalase, or indole, and its production of alkaline phosphatases. *H. haemoglobinophilus* ferments a number of carbohydrates. The ability of *H. ducreyi* to ferment carbohydrates needs further investigation.

The results of our biochemical and physiological studies agree with those reported by Kilian (9), except that we found that *H. ducreyi* produced alpha-hemolysis in stabs when grown on an RBA plate and was oxidase positive when tested on that medium. Three strains were urease positive, and CO<sub>2</sub> improved the growth of seven strains.

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