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Isolation and Cultivation of *Haemophilus ducreyi*

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A useful method for isolating and recognizing *Haemophilus ducreyi* from chancres and buboes of male patients is presented. A total of 41 clinical isolates of *H. ducreyi* were recovered from 33 patients over an 8-year period, and the experience with the 15 most recent isolates is presented in detail. Chocolate agar supplemented with 1% IsoVitaleX and 5% sheep blood agar were prepared, using Trypticase soy and Mueller-Hinton agar bases, and incubation conditions included ambient, capneic, and anaerobic environments. Mueller-Hinton agar was clearly superior over Trypticase soy agar for isolation of *H. ducreyi*, although there was little difference between 5% sheep blood and supplemented chocolate agar. Growth in ambient air and under anaerobiasis was poor or lacking, whereas growth in 5 to 7% CO_2 was good to luxuriant. Heat-inactivated and fresh (unheated) human blood clot tubes also were used for selective isolation. Although the rates of isolation from the two types of clot tubes were not significantly different, unheated clot tubes were superior to heated clot tubes because of reduced level of contaminants. Gram stain characteristics taken from blood clot tubes and solid media, cellular and colonial morphology of the bacilli, and lack of oxidase, catalase, and biochemical activity except nitrate reductase were determinant factors. The results of this study demonstrated that successful isolation of *H. ducreyi* can be achieved with a minimal amount of resources and expertise.

Haemophilus ducreyi causes chancroid in humans. Since other sexually transmitted diseases can mimic chancroid closely (2, 3, 11), it is essential to demonstrate *H. ducreyi* as the etiological agent in order to make the correct diagnosis. The isolation of *H. ducreyi* is often considered to be a difficult task, discouraging serious attempts to demonstrate the organism in ulcerations. Methods used to simplify the isolation of *H. ducreyi* have been sought by only a few workers, with isolation techniques focusing on the use of human (2, 4) and rabbit (7, 12) blood as well as selective media containing antibiotics (7, 11) for the recovery of *H. ducreyi*. Unheated human (4) and heat-inactivated human (2, 4) and rabbit (12) blood clot tubes have proven useful for isolation, although results are not in agreement as to the superiority of heated over unheated blood.

This report presents the methods used in our laboratory to isolate and identify *H. ducreyi*. It also describes the comparative methods which have proven useful for isolation and emphasizes the fact that no special media or elaborate techniques are required for the selective recovery of this organism.

MATERIALS AND METHODS

Source of isolates. Forty-one isolates of *H. ducreyi* were recovered during an 8-year period (1972 to 1980) in the Microbiology Laboratory, Madigan Army Medical Center, from specimens taken from 33 male patients suspected of having chancroid. Twenty-nine single isolates were from penile ulcers only, and two were from inguinal nodes only. *H. ducreyi* from both penile ulcers and inguinal nodes was isolated from two remaining patients. Follow-up cultures from penile ulcers of four patients and from the inguinal nodes of two other patients also yielded *H. ducreyi*. Because of (i) uniformity of isolation methods and (ii) more complete testing and recording of observations, this report includes the experience with only the 15 most recent isolates of *H. ducreyi*. Two representative strains were sent to the Centers for Disease Control, Atlanta, Ga., and confirmed as *H. ducreyi*.

Culture techniques. Clinical specimens were received as moistened swabs of penile lesions or as syringe aspirates of inguinal bobos. Eight to 10 ml of blood also was taken from each patient before antibiotic therapy and was divided equally into two sterile 13 by 100-mm screw cap test tubes. The freshly drawn blood was allowed to clot, the serum was aseptically removed, and one tube of the pair was inactivated at 56°C for 30 min.

The clot tubes were inoculated by adding a few drops of the aspirate to the serum-blood mixture or by gently swirling the swab in the serum surrounding the formed clot. Direct culture plates were inoculated with a few drops of the aspirate or by rolling the swab over one-quarter of each plate. The principal media used for isolation and cultivation were Mueller-Hinton sheep blood agar (MHBA; BBL Microbiology Systems, Cockeysville, Md.) and Mueller-Hinton chocolate agar

(MHCA) with 1% IsoVitaleX (BBL), contained in 150-mm plates. Thayer-Martin, neomycin sheep blood, mannitol salt, and MacConkey agars in 100-mm plates also were inoculated for the isolation of other pathogens. All plates were streaked for isolation, and a 5-μg methicillin and a 10-μg ampicillin disk were added to each MHBA and MHCA plate in the area of heavy inoculation. Thioglycolate broth was used for the isolation of anaerobes.

The tubes and plates were incubated at 35°C in an atmosphere of 5 to 7% CO_2 in an incubator with controlled humidity (ca. 85%). Plated media were examined daily for 7 days before being discarded as negative for *H. ducreyi*.

Culture examination. A Gram stain was prepared from each of the clot cultures after 24 h of incubation, and each tube was routinely subcultured to MHBA and MHCA. If no organisms suggestive of *H. ducreyi* were seen, a second Gram stain was prepared from each tube after an additional 24 h of incubation. Subcultures were made at this time only if suspicious organisms were seen which were not observed with the first Gram stain. The type and quantity of growth on all plated media were noted and recorded. During the subsequent daily examinations, particular attention was given to tiny colonies beginning to emerge (on the MHBA and MHCA plates) which were not apparent during earlier examination, either in the isolation area or within the zones of inhibition surrounding the antibiotic disks. Gram stains were made of suspicious colonies on the direct and subculture plates. Those colony forms consistent with *H. ducreyi* were subcultured to MHBA and MHCA for purity and for further study.

Identification of isolates. Organisms were identified by growth characteristics, by Gram stain morphology, and by oxidase, catalase, and biochemical reactions. Biochemical activity (1, 10) was determined with the Minitek system (BBL), using *Neisseria* broth, *Neisseria* broth containing 10% rabbit serum, and Trypticase soy broth (TSB; BBL) containing 5 and 10% supplement-C enrichment (Difco Laboratories, Detroit, Mich.) for supportive growth. The Minitek plates were incubated under CO_2 for 48 h. Conventional nitrate broth (0.2% KNO_3 in peptone broth) and nitrate broth containing 10 and 20% rabbit serum were used to test for nitrate reduction. A heavy suspension of each test organism was made in 0.5 ml of each nitrate medium and incubated at 35°C under CO_2 for 48 h before testing.

Factor requirements. Tests for X- and V-factor requirements were performed as previously described (10), using commercially available X- and V-factor disks (Difco) or strips (BBL).

Beta-lactamase test. Beta-lactamase production was assayed by the penicillin-starch paper strip method described by Jorgensen et al. (8).

Antimicrobial susceptibility. Antimicrobial susceptibility tests were performed by the agar disk diffusion method. Young, fresh cultures were suspended in TSB and adjusted to the 0.5 McFarland turbidity standard. The exact turbidity was difficult to achieve because of the granularity of *H. ducreyi*. The suspensions were streaked evenly over the surface of 150-mm MHBA plates, and the plates were allowed to dry for 15 min. Since zone diameters with many antibiotics approached 60 mm, not more than four well-spaced, high-potency antibiotic disks (BBL) were placed on each plate. The plates were incubated under 5 to 7% CO_2 in the humidified incubator. Final zone determinations were made after 48 or 72 h incubation, with the zones of inhibition measured with reflecting light. Minimal inhibitory concentration determinations were not attempted.

RESULTS

Between 1972 and 1976, a variety of protocols were followed to ascertain the most reliable means to recover the *H. ducreyi* bacillus. Since 1976, 15 patients were confirmed to have chancroid by isolation of the causative organism on solid media, and these strains are described below.

Isolation of strains. Table 1 shows the morphological and physiological characteristics of the 15 strains of *H. ducreyi*. Growth was taken from solid media, crushed on a slide, and stained by the Gram method. Whole intact microcolonies or fragments in multiple parallel chains of gram-negative rods appearing in "school of fish" formations or in "fingerprint" patterns due to multiple rows of chaining bacteria were seen. These microscopic features were characteristic for *H. ducreyi* and were diagnostic. Direct Gram stains prepared from clinical material were performed sparingly, since the procedure often was unreliable and the microscopic picture was potentially misleading.

Growth requirements. (i) Solid media. Agar media lacking blood products or supplements such as Trypticase soy agar (TSA), brain heart infusion agar, or Mueller-Hinton agar failed to support the growth of *H. ducreyi*. Conventional tests using X, V, and XV factors on TSA or Mueller-Hinton agar also failed to promote any growth. Growth of *H. ducreyi* was very poor on 5% sheep blood agar and chocolate agar with TSA base. Growth on MHBA and MHCA, however, was unsurpassed, with no perceptible difference in the quality or quantity of growth between the two media, the accessory growth factors provided by the supplemented chocolate agar notwithstanding.

(ii) Carbon dioxide. Selected isolates were subcultured to MHCA and incubated concurrently in ambient air, in 5 to 7% CO_2, and in an anaerobic environment (GasPak jar; BBL). To provide the necessary humidity requirements, the ambient air and anaerobic cultures were supplied with moistened gauze sponges. In all cases, there was essentially no growth under conditions other than CO_2. When a heavy inoculum was used, extremely slight growth was obtained with some strains under anaerobiosis or in ambient air after 4 or 5 days of incubation. No attempt was made to determine the optimal CO_2 concentration of any of the strains tested,

TABLE 1. Morphological and physiological characteristics of *H. ducreyi*

Characteristic	Result
Hemin requirement	No growth in absence.
CO_2 requirement	Enhanced growth in presence. Minimal or no growth in ambient air or under anaerobiasis.
Humidity requirement	Humid atmosphere required. Optimal growth on relatively fresh, moist medium.
Hemolysis	Initially nonhemolytic. Weak beta-hemolysis develops on Mueller-Hinton blood agar after 4 or 5 days.
Rate of growth	Slow. Single colonies not visible until 48 to 96 h of incubation.
Colony characteristics	Raised, opaque, compact, granular, tan or yellowish; difficult to pick up with a loop as colonies slide over agar surface; impossible to emulsify into a homogeneous suspension.
Gram stain morphology	Colonial fragments and intact microcolonies consisting of concentric wavy rings of multiple parallel chains of uniformly staining gram-negative rods. "School-of-fish" or "fingerprint" patterns may be observed.
Organism viability	Rapid deterioration (autolysis?) as observed by Gram stain; parallel chains appear to coalesce; typical morphology for only 1 to 2 days followed by amorphous debris (ghost forms).
Organism maintenance	Continuous subculture, as growth becomes visible, necessary to maintain viability on artificial media. Preserves well in sterile 2% skim milk at −70°C for at least 4 years.

although it is clear that the organisms require both O_2 and increased CO_2 levels for growth.

Use of blood clot tubes. Of 15 specimens that yielded *H. ducreyi* on solid media, Gram stains prepared from 9 corresponding blood clot tubes were positive for suspicious organisms after 24 h of incubation and from 3 additional tubes after 48 h of incubation. Although the direct inoculation plates recovered all 15 isolates, only these 12 clot tubes gave positive subcultures. There was heavy overgrowth of enteric bacteria on culture plates from two of the negative clot tubes and overgrowth of group B streptococci from the third.

There were no absolute differences in isolation of *H. ducreyi* from either the heated or unheated tubes, although there was less contamination resulting from subcultures of the unheated tubes. For example, with two specimens from which no *H. ducreyi* was isolated, organisms suggestive of *H. ducreyi* were seen by Gram stain from each of the heated tubes but not from the corresponding unheated tubes. These heated tubes grew out *Bacterioides melaninogenicus* upon anaerobic subculture, whereas the unheated tubes did not.

Biochemical reactions. Table 2 shows the biochemical characteristics of *H. ducreyi* and demonstrates the inactivity unique to these organisms. With the Minitek system, biochemical activity was absent, including the test for nitrate reduction. Conventional nitrate broth supplemented with 20% rabbit serum, however, yielded positive tests for nitrate reduction with all 15 strains. None of the strains reduced nitrate in the unsupplemented broth, and the results were inconsistent when nitrate broth supplemented with 10% rabbit serum was used.

Antimicrobial susceptibility. Table 3 presents the results of disk diffusion susceptibility tests. Eleven of the 15 isolates produced beta-lactamase, and the susceptibility to the penicillin-class antibiotics corresponded directly with this enzyme activity. Cephalothin was unaffected by beta-lactamase, and responses to gentamicin, streptomycin, and vancomycin were uniform. Of notable interest was the inverse relationship in the responses by three of the four beta-lactamase-negative strains to chloramphenicol and tetracycline in contrast to that seen with the penicillins. Of all the antibiotics tested, only erythromycin consistently showed large zones for all test strains.

DISCUSSION

The use of blood clot media has been advocated to enhance the isolation of *H. ducreyi* from clinical material. Deacon et al. (4) and Borchardt and Hoke (2) used heated human blood, and Teague and Deibert (12) used heated rabbit blood, for culturing. Deacon and associates (4) found that virulent *H. ducreyi* grew equally well in either fresh or heat-inactivated clots, whereas stock strains (avirulent by rabbit pathogenicity tests) grew only in inactivated clots. These findings suggested that previous investigators who determined that fresh blood was inhibitory for the strains tested based their conclusions on studies using avirulent or stock cultures.

The use of paired tubes has been limited to only the past 4 years, but our findings firmly support the conclusion of Deacon and associates (4) that there is a distinct disadvantage to the use of inactivated (heated) blood. *H. ducreyi* was isolated from fresh clots with a minimum of

TABLE 2. Biochemical characteristics of 15 strains of *H. ducreyi*

Substrate or test	Test result[a]
Oxidase	− (within 30 s)
Catalase	−
Acid from carbohydrates glucose, lactose, sucrose, maltose, xylose, mannitol, mannose, fructose	−
Nitrate reduction	+[b]
O-nitrophenyl-β-D-galactosidase	−
Decarboxylase (lysine, ornithine)	−
Dihydrolase (arginine)	−
Urease	−
Indole production	−
X, V, and XV factors (growth)	−

[a] Symbols: −, negative test; +, positive test.
[b] Results with nitrate broth supplemented with 20% rabbit serum.

contamination, whereas heated blood allowed contaminants to proliferate. Contaminants were undoubtedly suppressed in the fresh blood due to the bactericidal properties of the unheated blood, whereas the heat destroyed the complement necessary to inhibit the avirulent contaminants present.

A second disadvantage to the use of inactivated blood was that heating provided a favorable environment for the growth of anaerobic organisms present in chancroid-like lesions (3, 4, 7). *Bacterioides* species morphologically resemble *H. ducreyi*, and the presumptive diagnosis made by Gram stain from the inactivated blood could be highly misleading. The use of an unheated clot tube in the isolation protocol, however, although not necessary for the isolation of *H. ducreyi*, is a means of rapid presumptive diagnosis when the contents are Gram stained after overnight incubation, and it provides support to the isolation attempts.

Our early isolates of *H. ducreyi* were resistant to the penicillin-class antibiotics, and this prompted the use of methicillin and ampicillin disks for the selective isolation from contaminated plates. On several occasions when heavy mixed flora were present, success was achieved in isolating *H. ducreyi* only by picking colonies from within the zones of inhibition of the contaminants. Subsequently, penicillin-susceptible strains were encountered, which made this practice tenuous as a means of selective isolation. Hammond et al. (7) reported using vancomycin (3 μg/ml) in a selective isolation medium, with a more recent report confirming the efficacy of this agent (11). Our attempts to use low-potency (5 μg) vancomycin disks did not prove fruitful, since the zones of inhibition for *H. ducreyi* ranged from 12 to 18 mm, generally as large as or larger than the zones of gram-positive contaminants commonly present.

Our findings are in agreement with those of Sottnek et al. (11), who showed that Mueller-Hinton base was clearly superior to TSA base for isolation and cultivation of *H. ducreyi*. These workers showed, moreover, that hemin or growth factors present in fetal bovine serum are necessary for favorable growth regardless of the type of base medium chosen. *H. ducreyi* fails to grow in the vicinity of X disks or strips on unsupplemented TSA or Mueller-Hinton agar because these organisms require hemin in excess of that which is provided in the disks (5). It is significant that *H. ducreyi* would, indeed, grow around commercial X and XV strips on GC agar base (GIBCO Laboratories, Grand Island, N.Y.) when supplemented with glucose (0.1%),

TABLE 3. Antimicrobial susceptibility of 15 strains of *H. ducreyi* to 13 antibiotics[a]

Antibiotic	Beta-lactamase production (zone diam, mm)			
	Positive (11)[b]		Negative (4)	
	Range	Median	Range	Median
Penicillin	6	6	37–50	42
Ampicillin	6–15	8	40–50	43
Methicillin	6	6	9–30	17
Carbenicillin	15–30	20	50–58	53
Cephalothin	33–55	38	34–48	37
Gentamicin	25–37	29	28–32	31
Streptomycin	23–32	25	26–30	29
Erythromycin	45–60	55	54–60	56
Chloramphenicol	45–60	54	27–52	29
Tetracycline	18–26	21	12–20	12
Vancomycin	20–28	25	24–28	25
Colistin	6–12	8	8–14	12
Trimethoprim-sulfa-methoxazole	25–37	30	30–36	30

[a] Disk diffusion test on MHBA.
[b] Number in parentheses indicates number of strains tested.

glutamine (0.01%), and cysteine (0.05%) or with fetal bovine serum (11).

The only two biochemical characteristics manifested by *H. ducreyi* are reduction of nitrate to nitrate and production of alkaline phosphatase (5–7, 9). We were unable to demonstrate nitrate reduction with the Minitek system, which up to this time has been eminently suited to demonstrate this reaction by other *Haemophilus* species (1, 10). However, conventional nitrate broth supplemented with 20% rabbit serum gave positive tests when a heavy inoculum was used. The alkaline phosphatase test was not performed in our laboratory, but two representative strains submitted to the Centers for Disease Control for confirmation of identity were reported as positive for alkaline phosphatase.

The strains of *H. ducreyi* described in this report undoubtedly had their points of origin in the Far East, but it is not inconceivable that localized outbreaks similar to that which occurred in Winnipeg, Canada (7), could occur at any time. Isolation of the infective organism is the only positive means for definitive diagnosis of chancroid, and it is well within the scope of clinical microbiology laboratories to provide such cultural examination service. Laboratories routinely using MHBA or MHCA are well equipped to provide such service without resorting to special preparations or purchase of special media. Appropriate attention to detail in culturing techniques will heighten the probability of isolating *H. ducreyi* when present.

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LITERATURE CITED

1. **Back A. E., and T. R. Oberhofer.** 1978. Use of the Minitek system for biotyping *Haemophilus* species. J. Clin. Microbiol. **7**:312–313.
2. **Borchardt, K. A., and A. W. Hoke.** 1970. Simplified laboratory technique for diagnosis of chancroid. Arch. Dermatol. **102**:188–192.
3. **Chapel, T., W. J. Brown, C. Jeffries, and J. A. Stewart.** 1978. The microbiological flora of penile ulcerations. J. Infect. Dis. **137**:50–56.
4. **Deacon, W. E., D. C. Albritton, S. Olansky, and W. Kaplan.** 1956. VDRL chancroid studies. I. A simple procedure for the isolation and identification of *Haemophilus ducreyi*. J. Invest. Dermatol. **26**:399–406.
5. **Hammond, G. W., C. J. Lian, J. C. Wilt, W. L. Albritton, and A. R. Ronald.** 1978. Determination of the hemin requirement of *Haemophilus ducreyi:* evaluation of the porphyrin test and media used in the satellite growth test. J. Clin. Microbiol. **7**:243–246.
6. **Hammond, G. W., C. J. Lian, J. C. Wilt, and A. R. Ronald.** 1978. Antimicrobial susceptibility of *Haemophilus ducreyi*. Antimicrob. Agents Chemother. **13**:608–613.
7. **Hammond, G. W., C. J. Lian, J. C. Wilt, and A. R. Ronald.** 1978. Comparison of specimen collection and laboratory techniques for isolation of *Haemophilus ducreyi*. J. Clin. Microbiol. **7**:39–43.
8. **Jorgensen, J. H., J. C. Lee, and G. A. Alexander.** 1977. Rapid penicillinase paper strip method for detection of beta-lactamase-producing *Haemophilus influenzae* and *Neisseria gonorrheae*. Antimicrob. Agents Chemother. **11**:1087–1088.
9. **Kilian, M.** 1976. A taxonomic study of the genus *Haemophilus*, with the proposal of a new species. J. Gen. Microbiol. **93**:9–62.
10. **Oberhofer, T. R., and A. E. Back.** 1979. Biotypes of *Haemophilus* encountered in clinical laboratories. J. Clin. Microbiol. **10**:168–174.
11. **Sottnek, F. O., J. W. Biddle, S. J. Kraus, R. E. Weaver, and J. A. Stewart.** 1980. Isolation and identification of *Haemophilus ducreyi* in a clinical study. J. Clin. Microbiol. **12**:170–174.
12. **Teague, O., and O. Deibert.** 1920. The value of the cultural method in the diagnosis of chancroid. J. Urol. **4**:543–550.