

# Chancroid and *Haemophilus ducreyi*: an Update

DAVID L. TREES AND STEPHEN A. MORSE\*

*Division of Sexually Transmitted Disease Laboratory Research, National Center for Infectious Diseases,  
Centers for Disease Control and Prevention, Atlanta, Georgia 30333*

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## INTRODUCTION

*Haemophilus ducreyi* is a fastidious gram-negative bacillus that causes the sexually transmitted infection chancroid. There has been renewed interest in this pathogen and in chancroid since an association was demonstrated between genital ulcers and human immunodeficiency virus (HIV) infection. The state of our knowledge concerning the diagnosis and treatment of chancroid and the genetics, physiology, and pathogenesis of *H. ducreyi* was extensively reviewed in 1989 (5, 98). Both reviewers concluded that our knowledge was limited and that additional

studies were needed. In this article, we will summarize the progress that has been made during the past 5 years and highlight gaps in our knowledge of this interesting pathogen.

## EPIDEMIOLOGY

Chancroid is a genital ulcerative disease. These diseases are common throughout the world and include syphilis, genital herpes, chancroid, lymphogranuloma venereum, and donovanosis. Chancroid is particularly common in Africa, Asia, and Latin America, where its incidence may exceed that of syphilis as a cause of genital ulceration (109, 116). However, chancroid is considered an uncommon sexually transmitted infection in the United States. From data forwarded to the Centers for Disease Control and Prevention (CDC), the reported number of chancroid cases peaked in 1947 at 9,515 cases before begin-

\* Corresponding author. Mailing address: Centers for Disease Control and Prevention, MS G-39, 1600 Clifton Road, Atlanta, GA 30333. Phone: (404) 639-3559. Fax: (404) 639-3976.

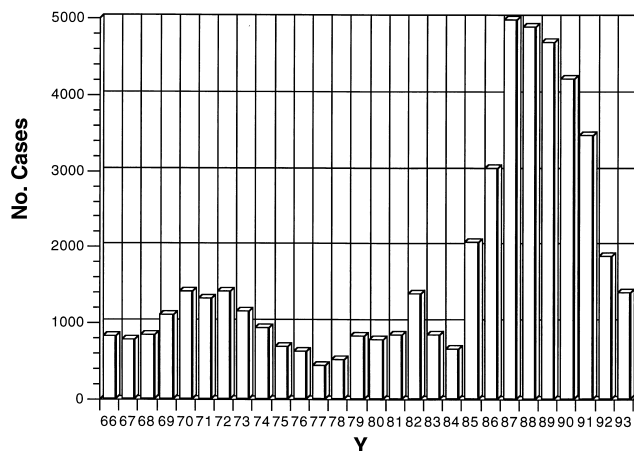


FIG. 1. Reported chancroid cases in the United States, 1966 to 1993.

ning a decline, which lasted until the mid-1980s. During the 20-year period between 1965 and 1984, the number of reported cases averaged 925 a year (range, 455 to 1,416 cases). However, beginning in 1985, the number of reported cases of chancroid increased dramatically, with 4,986 cases reported in 1987 (Fig. 1). Since 1987, the number of reported cases of chancroid has decreased steadily. Only 1,399 cases were reported to CDC in 1993 (34).

Chancroid cases are not evenly distributed throughout the United States. Most cases are reported among minorities living in eastern cities and in the South. In 1989, five states (Florida, Georgia, Louisiana, New York, and Texas) accounted for 94.7% of the reported cases of chancroid; however, the same five states accounted for only 82.8% of the reported cases of chancroid in 1993 (Table 1). This difference reflects a decrease in chancroid reports from these states coupled with an increase in areas where chancroid was formerly infrequently reported. Fourteen states did not report a single case of chancroid during the period from 1989 to 1993. Recent data suggest that chancroid is a common cause of genital ulcers in some areas of the United States. For example, *H. ducreyi* was detected in 37 of 101 consecutive men presenting at a New Orleans sexually transmitted disease (STD) clinic with genital ulcers (108).

It is likely that chancroid has been underreported in the United States. Many states reported only culture-confirmed chancroid cases, in part because a standard surveillance definition for a clinically compatible case was not implemented until 1990 (31). For surveillance purposes, a chancroid case may now be classified as either probable or confirmed. A probable case means the patient has clinically compatible symptoms, with one or more painful genital ulcers, and both (i) no evidence of *Treponema pallidum* infection by dark-field examination of ulcer exudate or by a serological test for syphilis performed at least 7 days after onset of ulcers and (ii) ulcers not typical of disease caused by herpes simplex virus (HSV) or a negative HSV culture. A case is confirmed when *H. ducreyi* has been isolated from a clinical specimen. However, the results of a recent survey of 115 STD clinics located in 32 states, Puerto Rico, and the District of Columbia (32) have indicated that culture medium for *H. ducreyi* was not always available and that these clinics had limited ability to differentiate among chancroid, syphilis, and genital herpes. Thus, in spite of the change in the case definition, chancroid cases are likely to be underreported. Future investigations, using state-of-the-art methods, are needed to elucidate the etiology of genital ulcers from different geographical sites.

The increase in the incidence of chancroid in the mid-1980s occurred at the same time that the incidence of primary and secondary syphilis increased among minority heterosexual men and women (34). The increase in syphilis was associated with cocaine use in both men and women (57, 130) and, among men, with the exchange of drugs or money for sexual favors (130). It was postulated that similar factors might also be responsible for the increase in chancroid. Recently, Martin and DiCarlo (93) reported that chancroid infection in male patients in New Orleans was strongly associated with crack cocaine use; however, drug use by the patient was really a marker for a more important risk factor: sexual exposure to a cocaine-using woman.

The persistence of chancroid in a population depends on several factors, which have been discussed in detail by Anderson (18). These factors can be expressed mathematically by the equation  $R_0 = \beta Dc$ , where  $R_0$  is the reproductive rate, defined as the average number of secondary cases generated by one primary case in a susceptible population of defined density;  $\beta$  is the average probability that the infection is transmitted per

TABLE 1. Reported cases of chancroid by state, 1989 to 1993<sup>a</sup>

State	No. of cases				
	1989	1990	1991	1992	1993
California	48	113	50	16	12
Florida	813	389	418	96	46
Georgia	295	324	76	21	21
Illinois	8	12	22	135	91
Louisiana	605	266	235	341	310
Massachusetts	46	27	2	13	2
New York	2,305	1,596	1,227	821	618
North Carolina	90	92	25	38	13
Ohio	2	16	7	7	21
Tennessee	0	1	70	39	8
Texas	619	1,303	1,273	319	37
Remaining states <sup>b</sup>	68	73	71	39	67
Total (% of U.S. total)	4,897 (98.7)	4,212 (98.3)	3,476 (98.0)	1,885 (97.9)	1,246 (94.6)

<sup>a</sup> Data taken from reference 34.

<sup>b</sup> Fourteen states (Hawaii, Alaska, Colorado, Idaho, Maine, Mississippi, Nebraska, Nevada, New Mexico, North Dakota, Oklahoma, Oregon, Pennsylvania, and South Dakota) reported no chancroid cases during the period 1989 to 1993.

sexual partner contact per unit time;  $D$  is the average duration of infectiousness of an infected individual; and  $c$  is the average number of sexual partners per unit time. If  $R_0 < 1$ , the infection will not persist and no epidemic will occur. Alternatively, if  $R_0 > 1$ , the infection will persist and an epidemic will occur. Over and Piot (110) estimated that the probability of transmitting chancroid from an infected male to an uninfected female during a single sexual exposure was 0.35, whereas the probability of transmitting chancroid from an infected female to an uninfected male during a single sexual exposure was 0.30. The duration of infectivity was estimated to be 45 days (110). The observation that some chancroid outbreaks in the United States have been associated with prostitution (21, 53) suggests that the number of sexual partners ( $c$ ) is a critical factor in the spread of chancroid. This may also help to explain the association of chancroid with certain risk factors, such as crack cocaine and alcohol use (93), since individuals who abuse cocaine or alcohol (17, 94, 143) have more sexual partners and are more likely to engage in high-risk sexual behavior (143).

STDs in general, and genital ulcerative diseases in particular, have been shown to be important risk factors in the heterosexual transmission of HIV (167). Genital ulcerative diseases as a clinical syndrome or as an etiological diagnosis were a significant risk factor for HIV seroconversion in prospective and cross-sectional case-control studies, even when sexual behavior was controlled for (26, 79, 119). The majority of these data come from studies performed in East African countries, such as Zaire and Kenya, where most genital ulcers are due to chancroid (118). However, similar associations were found in studies conducted in West Africa and in the Caribbean (56, 115). Two mechanisms have been proposed to explain how genital ulcers enhance the transmission of HIV. Chancroid and other genital ulcerative diseases could facilitate the transmission of HIV by increasing the shedding of virus through the ulcer. In fact, HIV has been detected in chancroidal ulcers (78, 120). The presence of an ulcer could also increase susceptibility to HIV infection by disrupting the epithelial barrier and perhaps by increasing the number of HIV-susceptible cells at the point of entry. CD4 T lymphocytes have been identified in lesions caused by *Treponema pallidum* (52) and HSV (37). Biopsy specimens of chancroidal lesions from persons experimentally infected with *H. ducreyi* revealed the presence of an infiltrate of T lymphocytes and macrophages in the dermis; the T lymphocytes expressed HLA-DR, indicating that they were activated (151). The proportion of these cells that are CD4 lymphocytes remains to be determined.

### CLINICAL FEATURES

The clinical features of chancroid and its varieties have been described previously (98). However, several recent reports suggest that prior HIV infection can modify the appearance and clinical course of chancroid. HIV-infected men tend to have a greater number of ulcers than those who are not infected with HIV (163), and those ulcers do not heal as readily. Quale et al. (123) reported the presence of a nonhealing penile lesion of 4 months' duration in a 31-year-old man infected with HIV. In addition, the patient had multiple nonhealing extragenital lesions on his leg and foot, which may have resulted from autoinoculation. An organism resembling *H. ducreyi* was isolated from the ulcer; however, the isolate was lost before confirmatory biochemical tests could be performed. The lesion responded to therapy with intramuscular (i.m.) ceftriaxone followed by oral erythromycin. No information on the extent of immunosuppression in the patient was given. HIV-infected patients may also have atypical lesions. Abeck et al. (1) re-

ported an elevated perianal ulcer, which was highly indurated, of 4 weeks' duration in a 25-year-old HIV-seropositive man. *H. ducreyi* was isolated from the lesion; the lesion was also positive by dark-field microscopy for *T. pallidum* and negative by culture for HSV. The patient had a CD4/CD8 ratio of 1.3 and a CD4 count of 350/mm<sup>3</sup>. Thus, he was only mildly immunosuppressed.

Concurrent HIV infection appears to modify the patient's response to chancroid therapy. Tyndall et al. (163) reported that among HIV-seropositive men with chancroid, therapy with a single dose of 250 mg of ceftriaxone i.m. failed more often than among men who were HIV seronegative. The HIV-infected men were thought to have only mild immunosuppression. In another study, which evaluated the treatment of culture-confirmed chancroid (87) with a single oral dose of the quinolone fleroxacin, bacteriological failure occurred in 3 of 11 HIV-infected men (27%), compared with 1 of 26 HIV-seronegative men (5%). In both of these studies, antibiotic resistance did not have a role in treatment failures, as the *H. ducreyi* isolates were highly susceptible to ceftriaxone (163) and fleroxacin (87). Thus, it is likely that other factors (e.g., immune status, pharmacokinetics, or the presence of a foreskin) are responsible for the high proportion of chancroid treatment failures among HIV-infected patients.

*H. ducreyi* has not been shown to cause systemic infection; however, extragenital lesions can occur and are thought to be the result of autoinoculation. It is interesting that disseminated *H. ducreyi* has not been reported, even in patients who are severely immunosuppressed by HIV infection. The relatively low optimum growth temperature (33 to 35°C) may preclude dissemination; however, further studies are needed to define the contribution of host immunity.

### TREATMENT

CDC recommends the following regimens (33) for the treatment of chancroid: azithromycin, 1 g orally in a single dose; ceftriaxone, 250 mg i.m. in a single dose; or erythromycin base, 500 mg per os (p.o.) four times a day for 7 days. Amoxicillin, 500 mg, plus clavulanic acid, 125 mg, p.o. three times a day for 7 days, and ciprofloxacin, 500 mg p.o. two times a day for 3 days, are alternative regimens. These alternative regimens have not been evaluated as extensively as the recommended regimens. The primary treatment recommended by the World Health Organization is erythromycin (500 mg p.o. three times a day for 7 days), with ceftriaxone (250 mg i.m. in a single dose), ciprofloxacin (500 mg as a single oral dose), spectinomycin (2 g i.m. as a single dose), and trimethoprim-sulfamethoxazole (TMP-SMX) (two tablets twice a day for 7 days) as alternative recommended regimens (170). Fleroxacin (400 mg as a single oral dose) has also been shown to be effective in the treatment of culture-proven chancroid (117, 164). Single-dose therapy (61, 117, 163) is advantageous because it avoids problems with compliance; however, cost is a major consideration in developing countries, where chancroid is prevalent.

Several factors affect treatment efficacy. As mentioned above, concurrent HIV infection was found to increase the probability of treatment failure following a single dose of either ceftriaxone (250 mg i.m.) or fleroxacin (400 mg p.o.) (87, 163); however, increasing the duration of fleroxacin treatment (400 mg orally once a day for 5 days) appears promising and should be studied further as a possible treatment regimen for HIV-infected men (164). In these studies, treatment failure was not due to infection with an antibiotic-resistant strain. However, antimicrobial resistance is extensive in *H. ducreyi* (see below), which will affect the choice of treatment regimen.

TABLE 2. Sensitivity of selective media for the isolation of *H. ducreyi*

Medium	Composition <sup>a</sup>	Sensitivity—no. positive/total (% positive)	Reference
GC-HgS	GC agar base (GIBCO) + 1% hemoglobin + 5% FBS + 1% IsoVitaleX (BBL) + vancomycin (3 µg/ml)	108/156 (69) <sup>b</sup>	41
MH-HB	Mueller-Hinton agar (BBL) + 5% chocolate horse blood + 1% IsoVitalex (BBL) + vancomycin (3 µg/ml)	143/178 (80) <sup>b</sup>	40
		63/156 (40) <sup>b</sup>	41
GC-HgS + MH-HB		126/178 (71) <sup>b</sup>	40
		110/156 (71) <sup>b</sup>	41
		150/178 (84) <sup>b</sup>	40
GC-FHB	GC agar base (GIBCO) + 5% Fildes' extract (Oxoid) + 5% horse blood + vancomycin (3 µg/ml)	133/178 (75) <sup>b</sup>	40
GC-FHBC	GC agar base (GIBCO) + 5% Fildes' extract (Oxoid) + 5% chocolate horse blood + vancomycin (3 µg/ml)	126/178 (71) <sup>b</sup>	40
HI-FRB	GC-HgS <sup>c</sup> + heart infusion agar (Difco) + 5% FBS + 5% fresh rabbit blood + 1% IsoVitaleX (BBL) + vancomycin (3 µg/ml)	25/35 (71) <sup>d</sup>	72
GC-HgS	CVA (GIBCO) substituted for IsoVitaleX	26/87 (29.8) <sup>e</sup>	88
MH-HB	CVA (GIBCO) substituted for IsoVitaleX	15/87 (17.2) <sup>e</sup>	88
GC-HgS + MH-HB	CVA (GIBCO) substituted for IsoVitaleX	56/87 (64) <sup>e</sup>	88
Sheffield	Sheffield medium + vancomycin (3 µg/ml)	0/87 (0) <sup>e</sup>	88
Sheffield	Sheffield medium + 5% defibrinated horse blood + vancomycin (3 µg/ml)	1/87 (1) <sup>e</sup>	88

<sup>a</sup> The manufacturer is given when the composition of the medium may vary with the source. GIBCO, GIBCO Diagnostics, Madison, Wis.; BBL, BBL Microbiology Systems, Cockeysville, Md.; Oxoid, United Kingdom; Difco, Detroit, Mich. FBS, fetal bovine serum.

<sup>b</sup> The patients were men with a clinical diagnosis of chancroid.

<sup>c</sup> GC agar base from Difco.

<sup>d</sup> The patients were 96 consecutive men with genital ulcers and no history of prior antimicrobial therapy. Sensitivity of culture was compared with the results of a PCR assay for *H. ducreyi* (72).

<sup>e</sup> The patients were 72 men and 15 women with dark-field-negative genital ulcers and no history of prior antimicrobial therapy.

Most isolates of *H. ducreyi* worldwide are susceptible to erythromycin, but strains for which the erythromycin MIC is 4 µg/ml have been isolated in Singapore (146) and Thailand (75). No resistance to ceftriaxone has been reported. Resistance to amoxicillin-clavulanic acid is more common among β-lactamase-negative strains than among β-lactamase-positive strains (75). One strain with a ciprofloxacin MIC of 2.0 µg/ml has been isolated in Thailand (75); however, quinolone resistance does not appear to be widespread. *H. ducreyi* resistance to TMP-SMX has been reported in Rwanda (166); the prevalence of resistant strains increased from 9% (2 of 23) in 1988 to 48% (34 of 71) of strains examined in 1991. All of the TMP-SMX-resistant isolates were resistant to TMP (MIC, >4.0 µg/ml). Strains resistant to TMP-SMX have also been isolated in the United States (75), Thailand (75), and Kenya (117). These data suggest that TMP-SMX, and possibly amoxicillin-clavulanic acid, should no longer be recommended for the treatment of chancroid. The prevalence and spectrum of antimicrobial resistance in *H. ducreyi* demand that caution should be exercised when advocating widespread use of an antibiotic for the treatment of chancroid unless clinical isolates are routinely monitored for resistance.

## DIAGNOSIS

### Culture Methods

**Culture.** *H. ducreyi* is a fastidious microorganism which is difficult to isolate from genital ulcer specimens (98). A number of factors may be responsible for the reported difficulty in isolating *H. ducreyi* on selective medium. The growth factor supplements that have been used (i.e., IsoVitaleX [BBL], Vitox [Oxoid], and CVA [GIBCO]) are similar in composition; the only difference is that CVA enrichment contains 25.0 g of cysteine HCl per liter, whereas IsoVitaleX and Vitox contain 25.9 g of cysteine HCl per liter. Thus, it is unlikely that the

growth factor supplement is responsible for differences in isolation rate (Table 2). Differences in nutritional requirements between strains may be important, as investigators who have compared *H. ducreyi* growth on several media noted that isolates may be grown on some but not all media (40, 88). The variation in the ability of a given medium to support the growth of *H. ducreyi* may be related to the nitrogen source (98). Selective media for the isolation of *H. ducreyi* usually contain 3 µg of vancomycin per ml to inhibit the growth of gram-positive bacteria (64). Strains of *H. ducreyi* which are inhibited by 3 µg of vancomycin have been observed in outbreaks of chancroid in the United States (73). However, this observation needs to be confirmed in other laboratories.

Inhibitory medium components or inhibitory products of *H. ducreyi* metabolism have also been reported. Inhibitory lots of fetal bovine serum, which is often added to *H. ducreyi* medium and most likely serves to bind inhibitory substances (86), have been reported (70). The degree of inhibition appears to be related to the antibiotic susceptibility of the *H. ducreyi* strain and could be due to antibiotics transferred from the mother to the fetal calf. Nevertheless, this report points out the need to screen lots of fetal bovine serum for the absence of substances that can inhibit the growth of *H. ducreyi*. Fetal bovine serum can be replaced by either activated charcoal (86, 157) or bovine albumin (51); fetal bovine serum cannot be replaced by newborn calf serum (157). The reason for the latter remains unknown. However, the ability to replace fetal bovine serum with 0.2% activated charcoal will markedly reduce the cost of these media and make them easier to prepare for use in developing countries. One caveat is that the effectiveness of charcoal-containing serum-free media in isolating *H. ducreyi* from clinical specimens has not been extensively compared with the effectiveness of serum-containing media.

Another important factor is the patient population used for the evaluation of selective media for the diagnosis of chan-



TABLE 3. Accuracy of clinical diagnosis of chancroid

Study (reference)	Patients (N)	Chancroid prevalence <sup>a</sup> (%)	Diagnostic accuracy (%)
Dangor et al. (38)	Males (240)	71	80
Fast et al. (54)	Males (100)	48	75
Sturm et al. (153)	Males (39), females (5)	22	53
O'Farrell et al. (107)	Males (100)	22	42
O'Farrell et al. (107)	Females (100)	15	57
Chapel et al. (35)	Males (100)	2	33

<sup>a</sup> Estimated by culture.

croid. Several investigators have inoculated specimens from men with a clinical diagnosis of chancroid onto selective media (38, 107). To calculate the sensitivity of culture, the numerator is the number of positive cultures, and the denominator is the number of patients with chancroid; however, the validity of this number depends on the accuracy of the clinical diagnosis. Making an accurate clinical diagnosis of chancroid is difficult and will be influenced, in part, by whether the clinical presentation is typical or atypical, whether the lesion is caused by *H. ducreyi* alone or has mixed etiology, and the experience of the clinician. A comparison of studies that evaluated the accuracy of a clinical diagnosis of chancroid (Table 3) suggested that diagnostic accuracy was related to the prevalence of the infection in the population. Moreover, interstudy comparisons of chancroid culture sensitivity among men with a clinical diagnosis of chancroid are difficult. For example, in two studies conducted by the same group of investigators, the sensitivity of a GC agar base medium supplemented with hemoglobin, fetal bovine serum, IsoVitaleX, and vancomycin (GC-HgS) varied from 69 to 80%, whereas the sensitivity of Mueller-Hinton agar supplemented with chocolate horse blood, IsoVitaleX, and vancomycin (MH-HB) varied from 40 to 71% (Table 2).

MacDonald et al. (88) evaluated the sensitivity of culture for *H. ducreyi* for men with genital ulcer disease who had negative results by dark-field microscopy and no history of prior antimicrobial therapy. However, dark-field microscopy is difficult to perform, and its sensitivity varies with the stage of the ulcer (42). In addition, *H. ducreyi* is sometimes isolated from lesions positive for *T. pallidum* by dark-field microscopy (38). It is generally accepted that a combination of two media, e.g., GC-HgS and MH-HB, is optimum for the isolation of *H. ducreyi*. However, no single study has used these media to compare patients with a clinical diagnosis of chancroid and those with lesions negative for *T. pallidum* by dark-field microscopy. The results from two other studies indicated that the use of a combination of GC-HgS and MH-HB for the isolation of *H. ducreyi* from men with a clinical diagnosis of chancroid had a sensitivity that varied between 70 and 84%, while a sensitivity of 64% was reported for men with dark-field-negative ulcers (Table 2). These differences may not be significant in light of the variables discussed above. More recently, Johnson et al. (72) compared the results of culture (GC-HgS and heart infusion agar supplemented with fetal bovine serum, fresh rabbit blood, IsoVitaleX, and vancomycin [HI-FRB]) for 96 consecutive men with genital ulcers and no history of prior antimicrobial therapy with the results of a PCR assay for *H. ducreyi*. Compared with PCR, culture had a sensitivity of 71% (Table 2). This value probably more accurately reflects the sensitivity of culture in an unselected population of men with genital ulcers.

**Transport medium.** Culture media for the isolation of *H. ducreyi* are often not available in peripheral laboratories be-

cause they are expensive to prepare and have a limited shelf life. The need for culture media at these sites can be overcome by the use of transport media that will maintain the viability of *H. ducreyi* until it is inoculated onto selective media at a central laboratory. Recently, Dangor et al. (41) compared the survival of a recently isolated strain of *H. ducreyi* in Stuart's, Amies', and four newly formulated thioglycolate-hemin-based transport media containing various combinations of selenium dioxide, albumin, and glutamine. No viable *H. ducreyi* were recovered when the inoculated transport media were held at room temperature (ca. 20°C) for 1 day; however, after 4 days at 4°C, viable *H. ducreyi* were recovered only from thioglycolate-hemin-based media containing either selenium dioxide, glutamine, and albumin or glutamine and albumin. These new transport media were subsequently evaluated (41) by using specimens from 156 patients who had been clinically diagnosed with chancroid. By direct plating on two different selective media, 110 of 156 (71%) specimens yielded positive cultures; duplicate swabs placed in transport media and held at 4°C for 4 days yielded 117 (75%) positive cultures when the specimens were inoculated onto the same media. Although recovery appears to be enhanced, several points bear mentioning. First, contamination appeared to be reduced after 4 days at 4°C; seven specimens that were contaminated on direct plating yielded *H. ducreyi* from the transport medium. Holding specimens at 4°C for 4 days resulted in an additional seven positive specimens that were originally negative on direct plating; however, five specimens that were initially positive were contaminated after 4 days at 4°C and another two were negative. These differences could be due to sampling error, as multiple swabs were obtained from each patient. Nevertheless, the use of these transport media may be a viable alternative when selective media are unavailable, if a reliable source of refrigeration exists.

### Nonculture Diagnostic Tests

**Polyclonal and monoclonal antibodies.** The development of assays for the direct detection of *H. ducreyi* in smears by immunofluorescence has been hindered, in part, by the poor specificity of polyclonal antisera and, until recently, by the unavailability of specific monoclonal antibodies (MAbs) (98). Denys et al. (46) prepared antiserum to *H. ducreyi* for use in an indirect immunofluorescence assay by immunizing rabbits intravenously with heat-killed cells. The use of an adjuvant was not mentioned. The polyvalent antiserum thus prepared had to be extensively absorbed with *Haemophilus* species to eliminate cross-reactivity. Even after absorption, it gave a moderate degree of fluorescence with various *Haemophilus* species, *Staphylococcus aureus*, and *Bacteroides melaninogenicus*. Since preimmunization sera were not obtained, it was not possible to assess whether cross-reacting antibodies were present prior to immunization. More recently, Odumeru et al. (105) immunized rabbits and mice with UV-killed cells of *H. ducreyi* emulsified in Freund's complete adjuvant. No reactivity with *H. ducreyi*, *Haemophilus influenzae*, or *Haemophilus parainfluenzae* was observed on immunoblots probed with preimmunization sera; however, substantial cross-reactivity was observed with whole-cell proteins from *H. influenzae* and *H. parainfluenzae* on immunoblots probed with rabbit or mouse anti-*H. ducreyi* antiserum. The cross-reactivity, which could be reduced by absorption with *H. influenzae* or *H. parainfluenzae*, was also apparent in a direct enzyme-linked immunosorbent assay (ELISA). It is also possible that the nature of the antigen or the immunization protocol may quantitatively or qualitatively affect the production of antibody to *H. ducreyi*. Finn et al. (58)

TABLE 4. Characteristics of MAbs to *H. ducreyi*

Reference	MAb	Class	Specific for <i>H. ducreyi</i>	Antigen
Campagnari et al. (28)	3F11	IgM	No	LOS
	6B4	IgM	No	LOS
Hansen and Loftus (66)	9D12	IgG2a	Yes	39–42-kDa OmpA-like MOMP
	8H4	IgG2a	?	14-kDa OMP
Spinola et al. (150)	2C7	NS <sup>a</sup>	No	39–42-kDa OmpA-like MOMP
Schalla et al. (141)	B3	NS	No	62-kDa OMP
Finn et al. (58)	A4	IgG2a	Yes	29-kDa OMP
	B11	IgG2a	Yes	29-kDa OMP
	D1	IgG2a	Yes	29-kDa OMP
Alfa et al. (16)	O83	NS	Yes	28-kDa protein
	O84	NS	Yes	28-kDa protein
	O86	NS	Yes	56-kDa protein
Hiltke et al. (68)	5C9	NS	Yes	28-kDa OMP
Spinola et al. (149)	3B9	IgG2a	No	18-kDa OMP
Hansen et al. (65)	1F8	NS	?	49-kDa OMP
	2H4	NS	?	23-kDa OMP

<sup>a</sup> NS, not stated.

prepared polyclonal antisera to *H. ducreyi* in rabbits by an immunization protocol, which involved subcutaneous inoculation of washed, live bacteria together with Freund's incomplete adjuvant, followed 2 weeks later by an i.m. injection of washed bacteria and then three daily intravenous inoculations of washed bacteria. The polyclonal serum thus obtained was evaluated by indirect immunofluorescence without prior absorption. *H. ducreyi* strains gave high immunofluorescence titers (160 to 1,280). The only cross-reactivity at a serum dilution of >1:20 was observed at a dilution of 1:80 with a strain of *Bordetella pertussis*. Antiserum prepared in this manner can potentially be used in antigen capture assays or in immunofluorescence assays.

A number of MAbs that react with antigens of *H. ducreyi* have been described (Table 4). Some of these MAbs appear to be species specific and to recognize surface-exposed determinants (e.g., 9D12, A4, B11, and D1). These MAbs may be suitable for direct detection of *H. ducreyi* in genital lesion smears. Karim et al. (74) evaluated an MAb (58) as an immunofluorescence reagent for the detection of *H. ducreyi* in genital lesion smears and in smears prepared from bubo aspirates from patients in Johannesburg, South Africa; Bangkok, Thailand; and Kuala Lumpur, Malaysia. Compared with culture, immunofluorescence had a sensitivity of 93% for ulcer specimens and 89% for bubo specimens. However, the specificity of immunofluorescence was only 63% for ulcer specimens and 56% for bubo specimens. The poor specificity likely reflects the insensitivity of culture performed on site.

Several MAbs react with epitopes on *H. ducreyi* that are also found on other bacteria (e.g., MAbs 3F11, 6B4, and 2C7). These MAbs will provide insight on structure-function relationships and help elucidate the role of cell envelope components in the pathogenesis of chancroid.

**DNA probes.** Parsons et al. (112, 113) reported the development of DNA probes for *H. ducreyi*. These investigators used high-titered polyclonal antiserum against formalin-killed *H. ducreyi* ATCC 33922 to screen an *EcoRI* *H. ducreyi* genomic library in  $\lambda$ gt11. Three DNA inserts coding for proteins that were recognized by the *H. ducreyi* antiserum were selected and

subcloned into a pUC13 plasmid vector. The <sup>32</sup>P-labeled probes, designated pLP1, pLP4, and pLP8, reacted strongly with 16 strains of *H. ducreyi*; no reactivity was observed with *T. pallidum*, *Neisseria gonorrhoeae*, or HSV DNA. The probes easily detected 10<sup>4</sup> CFU of *H. ducreyi*; weaker reactions were observed with 4.9 × 10<sup>3</sup> CFU, and negative reactions were obtained with 1.4 × 10<sup>3</sup> CFU. The probes also detected the presence of *H. ducreyi* DNA in rabbit lesion exudates; however, three of four positive reactions were obtained from culture-negative specimens. pLP8 also reacted weakly with relatively high numbers of a *Haemophilus* sp. and *Pasteurella* sp. (3 × 10<sup>7</sup> to 6 × 10<sup>7</sup> CFU); however, negative reactions were observed at lower cell concentrations (10<sup>5</sup> to 10<sup>6</sup> CFU). Subsequent sequencing of the insert in pLP8 (114) revealed that it encoded *H. ducreyi* homologs of *groE* and *groEL*. These are highly conserved genes and may explain the hybridization observed with the *Haemophilus* sp. and *Pasteurella* sp.

Two *H. ducreyi* probes have been developed by Chui et al. (36), based on published sequences of the 16S rRNA gene of *H. ducreyi* (48, 133). Although these probes were specific for *H. ducreyi*, neither reacted with all strains of *H. ducreyi* that were tested. DNA probes do not appear to have the sensitivity necessary for detecting the presence of *H. ducreyi* in clinical specimens. However, DNA probes have been used successfully for confirming the identification of *H. ducreyi* isolates (157).

**PCR.** PCR appears to offer a more sensitive and specific approach for the diagnosis of chancroid. Three PCR assays for chancroid have been developed. Chui et al. (36) used broad primers based on eubacterial 16S rRNA gene sequences to amplify a 303-bp sequence from members of the families *Pasteurellaceae* and *Enterobacteriaceae*. Using two *H. ducreyi*-specific probes internal to this sequence, they obtained 100% sensitivity with 51 strains from six continents that were isolated over a 15-year period. The clinical utility of PCR was compared with that of culture by using 100 clinical specimens from men with genital ulcers consistent with a clinical diagnosis of chancroid. Swab specimens were transported to the laboratory in phosphate-buffered saline containing chenodeoxycholate (1

mg/ml). After extraction, the DNA was amplified by means of a PCR protocol that involved 25 cycles of amplification, followed by use of Southern blot hybridization to detect the PCR product. A sensitivity of 83% and a specificity of 67% relative to culture were obtained. The sensitivity could be increased to 98% after three rounds of 25 cycles; however, the specificity compared with that of culture decreased to 51%.

Johnson et al. (71) developed a PCR assay with a pair of primers selected from the sequences of an anonymous fragment of DNA cloned from *H. ducreyi*. The 1,100-bp PCR product was detected on Southern transfers with a <sup>32</sup>P-labeled probe consisting of the entire cloned sequence. The specificity of the PCR assay was determined by using 118 isolates of *H. ducreyi* and 25 isolates belonging to related genera or organisms found in genital ulcers. No amplification was observed for any bacterium other than *H. ducreyi* with the exception of a single strain of *H. parainfluenzae*, which gave inefficient amplification of a 500-bp fragment that did not hybridize with the 1,100-bp fragment amplified from *H. ducreyi* DNA. The utility of this assay was assessed by using specimens from 217 consecutive patients who sought treatment for genital ulcers at an STD clinic. HSV cultures, dark-field examination, and syphilis serology were performed for each patient. Specimens for PCR were collected in the phosphate-containing transport medium described by Dangor et al. (41). Among a subgroup of 183 men whose genital ulcers contained a single etiologic agent (as determined by standard laboratory tests), PCR for *H. ducreyi* had a sensitivity of 65% and a specificity of 52% relative to culture. An 1,100-bp PCR product was amplified from the isolates of *H. ducreyi* obtained from culture-positive but PCR-negative ulcer specimens, indicating that sensitivity was not a problem. Further studies revealed the presence of *Taq* polymerase inhibitors in the preparations. The inhibitor was subsequently identified as P<sub>1</sub> (71, 72). For a second series of 96 men with genital ulcers, Johnson et al. (72) used the sample collection and preparation protocol described by Chui et al. (36) and obtained a PCR sensitivity of 100% and a specificity of 84% compared with culture. The decreased specificity likely reflected the poor sensitivity of culture.

Orle et al. (108) reported on the development of a commercial multiplex PCR assay that permits the simultaneous amplification of DNA targets from *H. ducreyi*, *T. pallidum*, and HSV types 1 and 2. This assay was evaluated on genital ulcer specimens from 101 consecutive patients. With respect to chancroid, 24 of 25 culture-positive specimens were positive by PCR. An additional 11 PCR-positive specimens were culture negative. A confirmatory PCR assay utilizing a different target gene suggested that these were not false-positive results. When commercially available, this PCR assay should improve the diagnosis of chancroid and other genital ulcer diseases.

#### SUSCEPTIBILITY TESTING AND ANTIMICROBIAL RESISTANCE

The methods and the results of susceptibility studies have been reviewed in detail elsewhere (39, 98). Most studies have used agar dilution; there has been little experience with disk diffusion susceptibility testing of *H. ducreyi*. Suffice it to say that susceptibility testing of *H. ducreyi* is not without problems. Dangor et al. (39) stated that the standards for antimicrobial susceptibility testing of fastidious organisms, including members of the genus *Haemophilus* (102), could not be applied to *H. ducreyi* because this organism requires hemin and supplementation with other nutrients for growth. Moreover, the cohesiveness of colonies and the agglutination of *H. ducreyi* in

suspension make it difficult to standardize inoculum size. The following procedures have been proposed (39) until a standardized agar dilution method for MIC determinations for *H. ducreyi* is developed: (i) homogenize colonies of *H. ducreyi* in Mueller-Hinton broth; (ii) mix the cell suspension in a Vortex mixer or by sonication and allow the suspension to stand for 15 min to allow clumps of organisms to settle; (iii) adjust the optical density to that of a 0.5 McFarland barium sulfate standard; (iv) use an enriched Mueller-Hinton agar (Mueller-Hinton agar base supplemented with 0.1% glucose, 0.01% glutamine, 0.025% hemin, and 5% lysed horse blood; GC agar base may be substituted for Mueller-Hinton agar base) at an incubation temperature of 35°C in a humid atmosphere containing 5% CO<sub>2</sub>; and (v) read plates after 48 h of incubation, with endpoints based on the lowest dilution of the antibiotic that yields no growth, two single colonies, or a fine, barely visible haze. For sulfonamide and TMP testing, a ca. 80% decrease in growth compared with that on the control plate may be used.

Geographical and temporal differences in antimicrobial susceptibilities have been observed. Many of the differences are due to the presence or absence of resistance plasmids (10, 100, 136, 137). Plasmids which encode resistance, either separately or in combination, to sulfonamides (6, 169), aminoglycosides (135, 169), tetracyclines (8, 92, 126–128), chloramphenicol (128), and β-lactam antibiotics (25, 89, 90, 124, 154) have been identified in *H. ducreyi* (Table 5). These plasmids have been reviewed in detail elsewhere (98). It is not unusual for a single *H. ducreyi* isolate to contain multiple resistance plasmids (45, 136, 137), including more than one plasmid that confers resistance to β-lactam antibiotics. Strains containing both a 7.0-MDa TEM-1 β-lactamase plasmid and a 3.5-MDa ROB-1 β-lactamase plasmid have been isolated frequently in Thailand (90).

The appearance of the class M tetracycline resistance determinant (Tet M) in *H. ducreyi* (126) is consistent with its presence in other genitourinary tract pathogens (127). The Tet M determinant is located on a 34-MDa conjugative plasmid. Unlike the Tet M-containing conjugative plasmid in *N. gonorrhoeae* (129), this plasmid is unable to mobilize Ap<sup>r</sup> plasmids (126). Tetracycline-resistant *H. ducreyi* strains have DNA sequences that exhibit homology throughout the length of the Tet M transposon, Tn916 (127); similar findings were observed with the genital tract microorganisms *Mycoplasma hominis* and *Ureaplasma urealyticum*. The source of the Tet M in *H. ducreyi* is unknown. However, since *Gardnerella vaginalis* and the 25.2-MDa plasmid of *N. gonorrhoeae* lack the complete transposon (127), it is unlikely that the Tet M originated in these microorganisms.

Very little information concerning the development of chromosomally mediated antimicrobial agent resistance in *H. ducreyi* is available. Approximately 30% of strains isolated in the United States between 1982 and 1990 were shown to lack resistance plasmids (100, 137). Some of these strains exhibited decreased susceptibilities to TMP, ciprofloxacin, and ofloxacin, suggesting the presence of chromosomally mediated resistance to these antimicrobial agents. MICs of penicillin G of 0.06 to 0.12 μg/ml have been reported for some strains of *H. ducreyi* (20), indicating in vitro activity by this class of antibiotics. However, increased MICs of penicillin G (0.25 to 1.0 μg/ml) have been observed in β-lactamase-negative strains of *H. ducreyi* (100), which is consistent with the development of chromosomally mediated resistance.



TABLE 5. Plasmids of *H. ducreyi*

Plasmid type	Mass (MDa)	Resistance determinant <sup>a</sup>	Characteristics <sup>b</sup>	Reference(s)
Antimicrobial resistance	3.1	Sm <sup>r</sup> Km <sup>r</sup>	Encodes two APH; enzyme modifying kanamycin appears to be a type I 3',5'-APH	135
	3.1	Su <sup>r</sup> Sm <sup>r</sup> Km <sup>r</sup>	Encodes type II Su <sup>r</sup> dihydropteroate synthase ( <i>SuII</i> ) and Sm <sup>r</sup> determinant, <i>strA</i> , similar to those found on plasmid RSF1010; Km <sup>r</sup> gene similar to that found on Tn903	50, 169
	3.2	Ap <sup>r</sup>	Encodes a TEM-1-type β-lactamase; identical to the 3.2-MDa gonococcal Ap <sup>r</sup> plasmid and carries about 40% of the TnA sequence	154
	3.5	Ap <sup>r</sup>	Encodes ROB-1-type β-lactamase; identical to the ROB-1 β-lactamase plasmid originally isolated in <i>Actinobacillus pleuropneumoniae</i>	90, 124
	4.9	Su <sup>r</sup>	Related to the enteric streptomycin and sulfonamide resistance plasmid RSF1010	6
	5.7	Ap <sup>r</sup>	Encodes TEM-1-type β-lactamase; homologous to the 3.2-MDa gonococcal Ap <sup>r</sup> plasmid and contains the complete TnA sequence; it differs from the 7.0-MDa Ap <sup>r</sup> <i>H. ducreyi</i> plasmid by the absence of a 1.3-MDa insertion element	25, 89, 154
	7.0	Ap <sup>r</sup>	Encodes a TEM-1-type β-lactamase; homologous to the 4.4-MDa gonococcal Ap <sup>r</sup> plasmid and contains the complete TnA sequence	25, 154
	30	Tc <sup>r</sup>	Conjugative plasmid; will not mobilize 7.0-MDa Ap <sup>r</sup> plasmid; appears to be related to a Tc <sup>r</sup> plasmid found in <i>H. influenzae</i>	8
	34	Tc <sup>r</sup> Cm <sup>r</sup>	Conjugative plasmid; can mobilize Ap <sup>r</sup> plasmids; 70–80% homologous to pRI234 from <i>H. influenzae</i> ; encodes a type II CAT and possesses a class B Tc <sup>r</sup> determinant	8, 92, 128
	34	Tc <sup>r</sup>	Conjugative plasmid; will not mobilize Ap <sup>r</sup> plasmids; carries most, if not all, of the TetM transposon	126, 127
Conjugative	23.5	None	Able to mobilize nonconjugative R plasmids; can cross species and generic lines; is distinct from the 24.5-MDa conjugative plasmid found in <i>N. gonorrhoeae</i>	45

<sup>a</sup> Su<sup>r</sup>, sulfonamide resistant; Sm<sup>r</sup>, streptomycin resistant; Km<sup>r</sup>, kanamycin resistant; Ap<sup>r</sup>, ampicillin resistant; Tc<sup>r</sup>, tetracycline resistant; Cm<sup>r</sup>, chloramphenicol resistant.

<sup>b</sup> APH, aminoglycoside phosphotransferase; CAT, chloramphenicol acetyltransferase.

## GROWTH AND NUTRITIONAL REQUIREMENTS

### In Vitro Growth

Very little is known about the growth and nutritional requirements of *H. ducreyi* (7, 98). Intercellular adhesion (98) is thought to be responsible for (i) the observation that colonies of this microorganism can be pushed intact across the surface of a solid medium and (ii) the clumps of cells that are observed in stained smears of *H. ducreyi* after growth on solid medium. The development of a defined liquid medium would facilitate studies on the growth and physiology of this microorganism. Recently, Dziuba et al. (51) studied the nutritional requirements of the type strain CIP 542 in a semidefined liquid medium by impedance technology. From the results of this study, they developed a simpler liquid medium, which contained the following (per liter): glucose, 10 g; NaCl, 6.67 g; NaH<sub>2</sub>PO<sub>4</sub> · H<sub>2</sub>O, 0.69 g; KCl, 0.20 g; MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.10 g; CaCl<sub>2</sub>, 0.10 g; L-glutamine, 0.10 g; L-serine, 0.10 g; and hemin, 0.005 g. The complex nitrogen source proteose peptone 3 (Difco), 2.0 g/liter, was required for growth; however, it could be replaced by either yeast extract (Difco), 2.0 g/liter, or gelatin (Difco), 17 g/liter. The utilization of these complex nitrogen sources was probably facilitated through the activity of a variety of aminopeptidases that are produced by this organism (98). Both glucose and glutamine were also found to be required for the growth of *H. ducreyi* (51). The doubling time of *H. ducreyi* incubated at 33°C under ambient atmospheric conditions was 2.4 to 2.5 h, as estimated from impedance data. However, if bovine serum albumin (0.1%, wt/vol) was added to the medium to absorb toxic metabolic products, the generation time decreased to 1.8 h.

Totten and Stamm (157) developed a clear broth and plate

medium which will be useful for physiologic and metabolic studies of *H. ducreyi*. The basal medium was identical in composition to GC agar base (Difco) and was supplemented with glucose and growth factors identical in concentration to those found in IsoVitalX enrichment (BBL); fetal bovine serum (10%, vol/vol) and catalase (0.10 g/liter). A doubling time of 3 to 4 h for *H. ducreyi* was calculated from absorbance readings of tubes of broth medium incubated at 35°C under microaerophilic conditions. The difference in doubling times observed by Totten and Stamm (157) and Dziuba et al. (51) could be due to differences in the medium composition, incubation conditions, or the method by which growth was measured. Nevertheless, the range of generation times (1.8 to 4 h) is considerably slower than the range of generation times for other bacteria (103) but is consistent with the observation that 48 to 72 h of incubation are often needed to produce visible colonies on solid media (98).

Clumping of *H. ducreyi* during growth on solid medium makes quantitation difficult. Accurate quantitation of cell numbers is required for studies on the pathogenesis of this organism. Totten and Stamm (157) observed that *H. ducreyi* strains grown in the clear broth medium reached a density of  $0.8 \times 10^8$  to  $2.5 \times 10^8$  CFU/ml in 24 h and, when examined microscopically, appeared as single cells or chains; however, when grown on solid medium, the same strains appeared as single cells and clumps when viewed microscopically. Other methods have been used to reduce clumping. Alfa et al. (13) harvested the growth from plates into broth, let the larger clumps settle by gravity, and removed material from the top of the tube, which contained single cells and small clumps. Dango et al. (41) found that suspending ca. 50 colonies of *H. ducreyi* in 2 ml of phosphate-buffered saline and passing the



suspension through a 28-gauge needle 8 to 10 times was an efficient way to disrupt clumps of cells. This method should be performed in a biological safety cabinet to avoid aerosols (99).

### Heme and Iron

Previous studies have demonstrated that *H. ducreyi* is unable to synthesize porphyrins or porphobilinogen from delta-aminolevulinic acid (7, 62). More recently, Lee (84) observed that the addition of protoporphyrin IX together with various sources of iron could not replace the requirement for heme. These results suggest that *H. ducreyi* lacks the enzyme ferrochelatase, which catalyzes the synthesis of heme by inserting ferrous iron into protoporphyrin IX. *H. ducreyi* is not closely related to other heme-dependent *Haemophilus* species (5, 98), which variably possess ferrochelatase (7). The results of earlier studies (7, 62) suggested that *H. ducreyi* required higher concentrations of heme than other *Haemophilus* species; 10 µg of heme per ml was required to initiate growth, whereas 200 to 500 µg of heme per ml was required for optimal growth. More recently, Dziuba et al. (51) observed that while 25 to 50 µg of hemin per ml was required for growth on solid medium, only 5 µg of hemin per ml was required for growth in liquid medium. These investigators postulated that decreases in the pH of the solid medium in the immediate area of a growing colony, resulting from the excretion of metabolic and end products, decreased the solubility of hemin and made higher concentrations necessary in order to compensate for the insoluble hemin.

The absence of ferrochelatase would explain the observations of Lee (84), who was unable to detect the production of siderophores by *H. ducreyi*. He observed that only hemin or the heme-containing proteins bovine hemoglobin, human hemoglobin, and bovine catalase (but not equine cytochrome  $C_{111}$ ) were capable of serving as the sole source of exogenous iron for the growth of *H. ducreyi*. Hemoglobin and heme, complexed with haptoglobin and serum albumin, respectively, could also be used as a source of iron. However, neither human transferrin, human lactoferrin, nor  $FeCl_3$  would support the *in vitro* growth of this microorganism.

The mechanism by which *H. ducreyi* acquires iron from catalase and hemoglobin is unknown. However, from observations in other systems, it is likely that catalase and hemoglobin interact with the cell surface of *H. ducreyi* (97) and that the intact heme molecule is internalized. Heme is associated through hydrogen bonding to hemoglobin and catalase (59), but it is covalently bound in cytochrome *c*. Thus, the ability of heme-containing proteins to serve as sources of heme appears to be correlated with the noncovalent attachment of heme.

The iron (heme) sources utilized by *H. ducreyi* (84) are primarily intracellular. *H. ducreyi* may gain access to these sources by cell invasion (83) or through the activity of a hemolysin (111).

### Selenium

Slootmans et al. (145) examined compounds that might improve the selectivity of the primary isolation medium for *H. ducreyi*. They observed that sodium selenate (MIC for 90% of strains [ $MIC_{90}$ ] = 4 µg/ml) but not potassium selenite ( $MIC_{90}$  = 500 µg/ml), selenomethionine ( $MIC_{90}$  = 750 µg/ml), or selenocystamine ( $MIC_{90}$  = 64 µg/ml) markedly inhibited the growth of *H. ducreyi* under ambient atmospheric conditions. Subinhibitory concentrations of selenium appeared to promote the growth of *H. ducreyi*. Vanden Berghe (165) observed that, under microaerophilic conditions, sodium selenite (0.1 µg/ml) added to a medium comprised of Mueller-Hinton agar, hemin, glutamine, and bovine albumin stimulated the growth of *H.*

*ducreyi*. However, Dziuba et al. (51) observed that the presence of sodium selenite in a liquid medium incubated under ambient atmospheric conditions did not stimulate the growth of *H. ducreyi* CIP 542. The reasons for these differences are unknown.

### STRAIN TYPING

Previous studies which used sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of outer membrane proteins, immunofluorescence, and enzyme profiles to phenotypically characterize strains of *H. ducreyi* were discussed in a previous review (98). *H. ducreyi* has very limited biochemical activities that can be used to characterize individual strains. The development of a typing system with a high degree of discrimination would have broad applicability to studies on *H. ducreyi*. Such a typing system could address questions concerning the geographical distribution of strains and mode of transmission, discriminate between treatment failure and reinfection, identify strains of differing virulence, and provide a means to study the genetic diversity of this interesting pathogen.

### Ribotyping

Ribotyping is based on restriction fragment length polymorphisms of rRNA genes, which are highly conserved and are usually present in multiple copies on the genome. Sarafian et al. (139) examined the *Hind*III and *Hinc*II banding patterns (ribotypes) of 44 strains after separating the fragments by agarose gel electrophoresis, transferring them to a nylon membrane, and hybridizing them with a  $^{32}P$ -labeled probe of 16S and 23S RNA from *Escherichia coli*. *Hind*III digests yielded eight ribotypes, whereas *Hinc*II digests yielded four ribotypes. Further studies on 132 strains from various geographical areas increased the number of *Hind*III ribotypes to 12 and the number of *Hinc*II ribotypes to 5 (138). The feasibility of using ribotyping for epidemiologic investigations of chancroid was suggested by the finding that four *Hinc*II and five *Hind*III ribotypes were observed among 14 *H. ducreyi* isolates collected during a 1-month period in Kenya, where chancroid is endemic.

Brown and Ison (23) used a digoxigenin-labeled cDNA probe prepared from *E. coli* 16S and 23S rRNA to circumvent the problems associated with using  $^{32}P$ -labeled probes. They identified 10 *Hind*III ribotypes and three *Hinc*II ribotypes among 30 strains of *H. ducreyi* from diverse geographic areas. However, maximum discrimination among these strains was obtained with *Bst*EII and *Bgl*II, which yielded 7 and 10 ribotypes, respectively. Both studies (23, 139) found that ribotyping was reproducible and that it discriminated among strains of *H. ducreyi*.

Rossau et al. (133) amplified part of an rRNA cistron of the type strain (CIP542<sup>T</sup>) by PCR with primers complementary to conserved regions in the 16S and 23S rRNA genes. With this technique, a 2,400-bp rRNA gene fragment was obtained, which, when sequenced, contained 1,498 bases of the 16S gene and 456 bases of the 23S gene. Restriction sites for the restriction enzymes used in ribotyping, i.e., *Hind*III, *Hinc*II, *Bst*EII, and *Bgl*II, were identified within these sequences (23).

Flood et al. (60) used ribotyping and plasmid analysis to investigate an outbreak of chancroid in San Francisco. From 1989 through May 1991, 54 cases of culture-confirmed chancroid were reported. Among the 32 isolates available for ribotyping, six different *Hind*III patterns were identified. Two ribotypes, *Hind*III-2 (four strains) and *Hind*III-11 (one strain),

were isolated from patients who had recently traveled to Los Angeles, Korea, or El Salvador. The *HindIII*-2 strains could be further differentiated on the basis of their plasmid profile; the strains isolated from the three patients who reported travel to Los Angeles contained a 5.7-MDa plasmid, whereas the strain isolated from the patient who traveled to Korea contained 1.9-, 2.6-, 2.9-, and 3.2-MDa plasmids. This plasmid pattern is similar to that observed in strains isolated in the Far East (136). Four ribotypes appeared to be acquired locally. The most interesting feature of this investigation was the finding of more than one strain of *H. ducreyi*, as defined by ribotype, with evidence indicating that some strains were acquired during travel outside the San Francisco area. In contrast, during a large outbreak in Orange County, California, all strains were found to possess a common 3.2-MDa plasmid and were presumed to represent a single strain (21). However, a retrospective analysis of 28 isolates from this outbreak revealed the presence of two *HindIII* ribotypes, suggesting that more than one strain may have been involved in this outbreak (139).

### Plasmid Analysis

Plasmid profiles have been used to gain insight into the distribution and extent of temporal changes in *H. ducreyi* strain populations in the United States (137). Five plasmid profiles have been identified among 342 strains isolated in 18 urban areas of the United States between 1981 and 1990. Isolates either were plasmidless (102 of 342) or possessed a 5.7-MDa plasmid (174 of 342), both a 4.4- and a 5.7-MDa plasmid (21 of 342), a 3.2-MDa plasmid (44 of 342), or a combination of the 1.8-, 2.6-, 2.8-, and 3.2-MDa plasmids (1 of 342). Although plasmid analysis provides some helpful information, its use as the sole typing system has significant limitations. Different strains may have identical plasmid profiles (60). Plasmids may also be lost or transferred by conjugation (98). Thus, plasmid profiles should be used in conjunction with another typing system (e.g., ribotyping) to provide additional discrimination (60).

### Immunotyping

Roggen et al. (130) examined the antigenic diversity within a panel of 63 *H. ducreyi* isolates by Western blot (immunoblot) analysis with pooled antisera from 238 patients with culture-proven chancroid. After absorption with a pool of soluble antigens from *H. influenzae*, *H. parainfluenzae*, *Haemophilus parahaemolyticus*, *Pasteurella ureae*, *N. gonorrhoeae*, *E. coli*, and *Actinobacillus actinomycetemcomitans* to remove cross-reacting antibodies, antigenic determinants more specific for *H. ducreyi* were identified. An immunodominant 28- to 28.5-kDa protein was expressed by all *H. ducreyi* isolates; 56 of 63 isolates revealed a dominant protein with a variable molecular mass of between 30 and 34 kDa. When both of these proteins were used as immunotypic markers, seven different immunopatterns or types were identified. Antigenic diversity was observed among isolates from different geographical areas as well as within a single area. How this typing correlates with ribotyping (23, 139) remains to be determined.

### Lectin Agglutination

Because lectins have the ability to bind to a wide variety of microbial components that contain either simple or complex carbohydrates, they can be used to differentiate between strains of bacteria on the basis of variations in cell surface carbohydrates. Korting et al. (77) tested the ability of 43 *H. ducreyi* isolates from geographically diverse areas to aggluti-

nate with a commercially available panel of 14 lectins (E. Y. Laboratories, San Mateo, Calif.) of known specificity. Each strain reacted with at least two lectins, and the majority reacted with more than five lectins. Among the strains tested, 20 different agglutination patterns were observed. Schalla and Morse (140) examined 63 isolates of *H. ducreyi* obtained from outbreaks in California, Georgia, New York, and Massachusetts, using the same commercially available lectin panel; the specificity of the agglutination reaction was assessed with specific carbohydrates. The isolates fell into one of two agglutination patterns, based on reactivity with the *Griffonia simplicifolia* II lectin; 57% of the isolates agglutinated with this lectin, and 43% of the isolates did not. There was no relationship between agglutination pattern and geographic location. In contrast to the results of Korting et al. (77), no agglutination was observed with the lectins of *Dolichos bifloris*, *Ulex europaeus* I, and *Lotus tetragonolobus*. Schalla and Morse (140) also noted that agglutination with lectins from *Lens culinaris* and *Phaseolus vulgaris* could not be inhibited with specific carbohydrates and was due to nonspecific clumping of the organisms. Korting et al. (77) reported that most of their isolates were agglutinated by these lectins; however, specificity controls were not included in this study. Lectin typing of *H. ducreyi* is of doubtful value, based on limited ability to discriminate between isolates, nonspecific agglutination reactions, and the absence of data concerning the stability of the carbohydrate-containing cell surface markers.

### HUMAN IMMUNE RESPONSE AND SEROLOGY

Delayed-type hypersensitivity to *H. ducreyi* antigens and the complement fixation test were formerly used as aids in the diagnosis of chancroid (67, 76). However, their poor sensitivity and specificity preclude their current use in diagnosis. Increased knowledge of the antigenic composition of *H. ducreyi* and the human immune response to infection has led to the development and evaluation of serologic tests for chancroid (Table 6). However, it is difficult to compare the results obtained with these tests because each test is different. Table 6 shows that strains as well as antigen preparations, sorbents, solid phases, serum dilutions, immunoglobulins (Igs) measured, development time and temperature, and the chromogen differed between assays. These assays have not been compared against one another with the same serum specimens. Nevertheless, some general conclusions can be derived from these studies. Circulating IgG (47, 101, 131, 141), IgM (47, 131, 141), and IgA (131) are present in patients with culture-confirmed chancroid. The specificity of these tests is improved by the removal of cross-reacting antibodies by absorption (14, 15, 47, 131); the *H. ducreyi*-specific antibodies are to the outer membrane protein (14, 131) and lipooligosaccharide (LOS) (14) antigens. A number of factors which affect the sensitivity of these tests and render their use in diagnosis problematic have been identified. The sensitivity of the test is reduced with acute-phase serum specimens (47) and in younger patients (131); however, sensitivity is increased with increased duration of infection (131). The sensitivity of the test is also antigen dependent (14, 15, 101, 131). The anti-*H. ducreyi* immune response remains elevated for extended periods of time (15); this is due almost exclusively to IgG. This extended antibody response will affect the performance (i.e., positive predictive value) of these assays in populations with a high prevalence of chancroid. HIV infection also affects the test performance; enhanced specificity of IgG and sensitivity of IgM assays for anti-*H. ducreyi* antibodies have been observed with serum specimens from HIV-positive patients (47). These assays have been used successfully to measure the prevalence of chancroid in

TABLE 7. Comparison of in vitro studies of *H. ducreyi* adherence, invasion, and CPE

Cells <sup>a</sup>	Reference	Adherence	Invasion	CPE	Time CPE observed
HEp-2 (T)	82	+	±	+	24-48 h
	122	NT <sup>b</sup>	NT	+	24-48 h
	155	+	+	NT	
Hec1B (T)	83	+	+	-	
HeLa (T)	144	+	+	+	3 days
	83	+	-	-	
	82	+	-	+	24-48 h
	122	NT	NT	+	24-48 h
HFF (Nt)	83	+	+	+	5 days
	13	+	-	NT	
	69	+	-	+	24 h
FS2-3 (Nt)	11	+	-	+	3 days
Vero (T)	2	NT	NT	-	
	122	NT	NT	-	
HFEC (Nt)	155	+	+	NT	
Human keratinocytes (Nt)	2	NT	NT	-	
	122	NT	NT	-	
	22	+	NT	NT	
AS49 (T)	122	NT	NT	+	24-48 h
	11	+	-	+	3 days

<sup>a</sup> T, transformed cell line; Nt, nontransformed cell line.  
<sup>b</sup> NT, not tested.

various individuals or groups to target and evaluate control efforts, as part of larger interventions designed to reduce the transmission of HIV.

**MODELS FOR THE STUDY OF *H. DUCREYI* PATHOGENESIS**

**In Vitro Studies**

Numerous in vitro models have been used to study *H. ducreyi* pathogenesis (Table 7). Alfa (11) compared the ability of *H. ducreyi* to cause a cytopathic effect (CPE) on adult foreskin cells (FS2-3, a primary cell line consisting mainly of fibroblasts) and human lung carcinoma cells (A549, a transformed cell line). Microscopic examination revealed that *H. ducreyi* formed “fungus-like” microcolonies on the FS2-3 cells but did not appear to invade these eukaryotic cells. Both *H. ducreyi* strains R018 and 35000 produced CPE on FS2-3 cells; no CPE was observed when *H. influenzae* and the “avirulent” *H. ducreyi* strain CIP542 were incubated with FS2-3 cells under similar conditions. None of the strains examined produced CPE on A549 cells. In another study, Alfa et al. (13) evaluated the attachment of “virulent” versus “avirulent” strains to human foreskin cell cultures by using <sup>125</sup>I-labeled *H. ducreyi*. The results showed that the virulent *H. ducreyi* strain 35000 attached to human foreskin cells at significantly higher levels, at both 22 and 35°C, than did the avirulent strain A77. Microscopic examination of the cell cultures suggested that *H. ducreyi* penetrated between the cultured foreskin cells; however, invasion of the cells was not evident. This group has also recently described the use of a chemiluminescent ribosomal probe method for determining the level of attachment of *H.*

TABLE 6. Comparison of enzyme immunoassays used for the detection of antibody to *H. ducreyi*

Reference	Source of antigen	Antigen	Absorption <sup>a</sup>	Microtiter plates	Serum dilution	Ig measured	Enzyme and chromogen <sup>b</sup>	Development time (min)/temp (°C)
101	Strain 35000	Particulate cell fraction	No	Type not specified (NUNC)	1:200	IgG	HRP with <i>o</i> -phenylenediamine; optical density measured at 492 nm	20/37
131	Pool of 9 strains representing immunotypes described in reference 87	Whole-cell lysate after sonication	Yes (A)	Maxisorb Immunoplate (IF96; NUNC)	1:200	IgG, IgA, IgM	HRP with <i>o</i> -phenylenediamine; optical density measured at 490 nm	10/RT <sup>c</sup>
15	Strain 35000 or strain R018	Whole-cell lysate after sonication	Yes (B)	Type not specified (Dynatech)	1:100	Total Igs	HRP with ABTS; optical density measured at 405 nm <sup>d</sup>	15/RT
47	Strain 35000	Soluble antigens after sonication	Yes (C)	Polysoorb Immunoplate (U96; NUNC)	1:200 and 1:400	IgG, IgM	HRP with ABTS; optical density measured at 405 nm	25/20
14	Strain 35000 or strain R018	Purified LOS or OMP preparation	Yes (B)	Immunon I (Dynatech)	1:100 (OMP), 1:200 (LOS)	Total Igs	HRP with ABTS; optical density measured at 405 nm	15/RT

<sup>a</sup> Absorption was done with (A) pooled antigens from *H. influenzae*, *H. parainfluenzae*, *Haemophilus parahaemolyticus*, and *E. coli*; (B) pooled antigens from *H. influenzae* and *H. parainfluenzae*; or (C) pooled antigens from *H. influenzae*, *H. parainfluenzae*, and *H. parahaemolyticus*.  
<sup>b</sup> HRP, horseradish peroxidase; ABTS, 2,2'-azino-bis(3-ethylbenzothiazolinesulfonic acid).  
<sup>c</sup> RT, room temperature.  
<sup>d</sup> Manufacturer recommends measurement at 415 nm.



*ducreyi* to eukaryotic cells (12). Lagergard et al. (82) used viable cell counts and radioassays to examine the ability of 10 *H. ducreyi* strains to attach to HEP-2 and HeLa cells. All 10 strains were observed to attach to both cell lines, with peak adherence occurring at 2 to 3 h. Gentamicin was added to HEP-2 cells in order to distinguish between attachment and invasion. The survival of a small percentage of the inoculum after gentamicin treatment suggested that some of the bacteria may be intracellular. These authors also observed eukaryotic cell death following prolonged incubation with *H. ducreyi*, suggesting that a cytotoxic product was being released by the bacteria.

Lammel et al. (83) observed both attachment to and invasion of eukaryotic cells by *H. ducreyi*. By electron microscopy, *H. ducreyi* strains 90-244 and CIP542 were shown to attach to HeLa 229, Hec1B, and HFF cells. Cells of strain 90-244 appeared as adhered whorls on the eukaryotic cells, whereas cells of strain CIP542 appeared as organized clumps. Using a gentamicin survival assay and electron microscopy, the authors demonstrated rapid invasion of HFF and Hec1B cells by strain 90-244; after 2 h, *H. ducreyi* cells were partially surrounded by what appeared to be a phagocytic membrane. Strain CIP542 was observed in the interstitial spaces between HFF cells as well as in the cytoplasm of these cells.

Shah et al. (144) also reported the presence of *H. ducreyi* within various epithelial cell lines. They noted that the presence of large numbers of intracellular organisms resulted in cell lysis and subsequent release of *H. ducreyi*. Totten et al. (155) examined the ability of various *H. ducreyi* to attach to and invade human foreskin epithelial cells (HFECs). Of the strains tested, six exhibited high levels of attachment to HFECs and two strains exhibited low levels of attachment. The strains that exhibited low levels of attachment were also avirulent in a rabbit infection model. The strain with the highest level of attachment was observed to invade the HFECs by ingestion into vesicles followed by release into the cytoplasm.

A recent study by Brentjens et al. (22) determined that *H. ducreyi* also binds to cultured human keratinocytes. After 2 h, 15 to 23% of the *H. ducreyi* inoculum was bound to the cell monolayer, compared with less than 1% for *E. coli* or *Moraxella catarrhalis*. This binding may be relevant to pathogenesis, since keratinocytes are probably the first host cells encountered by *H. ducreyi*.

### In Vivo Studies

For many years, the standard animal model for determination of *H. ducreyi* virulence was the rabbit intradermal model (49, 55). This model has several drawbacks, with the most outstanding being that the lesions do not resemble those of human chancroid. In recent years, studies have focused on developing better animal models that would be suitable for the in vivo study of *H. ducreyi* pathogenesis.

In 1988, Tuffrey et al. (161) reported that a mouse model of *H. ducreyi* infection had been developed in which pustules and ulcers similar to those seen in human infection were produced. However, later studies (162) showed that these lesions were formed following the injection of either viable or heat-killed *H. ducreyi* or with *N. gonorrhoeae*, which suggests that the lesions were not produced specifically by viable organisms or by *H. ducreyi*. Campagnari et al. (29) also observed that both viable and heat-killed bacteria produced lesions in rabbits as well as in mice, although viable organisms could not be isolated from the site of inoculation. Intradermal injection of 20 µg of purified LOS from either *H. ducreyi* or *N. gonorrhoeae* produced lesions in rabbits that were histologically similar to those pro-

duced following the injection of viable or heat-killed bacteria (29). Lagergard (80) also observed that intradermal injection of *H. ducreyi* LOS produced lesions in both mice and rabbits, although the amount of LOS required to consistently produce ulceration was approximately 200 µg.

Trees et al. (159) adapted the chambered mouse model developed by Arko (19) to study *H. ducreyi* pathogenesis. Subcutaneous polyethylene chambers implanted in mice were inoculated with various strains of *H. ducreyi*. Growth of *H. ducreyi* occurred within the chambers, with some mice maintaining the infection for as long as 4 months. No dissemination of *H. ducreyi* from the chamber was observed. When this model was used, variation in *H. ducreyi* proteins during in vivo growth was observed (160).

Purcell et al. (121) postulated that the rabbit's high body temperature might prevent the in vivo growth of *H. ducreyi*, which has an optimum temperature for growth of 33 to 35°C. Rabbits that were housed at 15 to 17°C in order to reduce skin temperature developed necrotic lesions following the intradermal inoculation of 10<sup>5</sup> CFU of *H. ducreyi*. Animals that were housed at ambient temperatures (23 to 25°C) did not develop lesions when inoculated intradermally with the same concentration of *H. ducreyi*. Lesion formation was dependent on *H. ducreyi* viability, and live organisms could be recovered from the lesions. Histopathologic examination of lesions demonstrated that the infection evolved from acute inflammation to abscess formation. Although the lesions observed were not identical to those seen in humans with chancroid, this model represents a marked improvement over previous rabbit models.

Recently, Totten et al. (156) developed a primate model to study chancroid pathogenesis. In this study, adult pigtailed macaques (*Macaca nemestrina*) were inoculated with 10<sup>7</sup> to 10<sup>8</sup> CFU of *H. ducreyi*. The sites of inoculation were the foreskin for male macaques and the vaginal labia for female macaques. Male macaques developed lesions that were similar in appearance and progression to those seen in human chancroid, with ulcers developing in 6 to 12 days. Viable *H. ducreyi* could be isolated for up to 20 days, and humoral antibodies, as assessed by Western blotting, were detected beginning 1 week after infection. Antibodies reacting to proteins with similar migration, as shown by SDS-PAGE and immunoblotting, have been detected in humans (98). None of the female animals that were inoculated with *H. ducreyi* developed visible ulcers and therefore were not evaluated for development of humoral antibodies. This lack of ulcer formation may be due to differences in the epithelium between males and females. The possibility of asymptomatic infection suggests that serologic studies should also be performed in the female macaques. Nevertheless, this model appears to be useful for the study of *H. ducreyi* pathogenesis in males.

### Human Studies

There have been no human challenge studies with *H. ducreyi* for almost 40 years (43). Recently, Spinola et al. (151) reported a study whose goal was to develop a safe and reliable *H. ducreyi* infection model in humans. Four volunteers were inoculated, by means of a multitest applicator, on the exterior surface of the upper arm. Three subjects received 10<sup>4</sup> to 10<sup>6</sup> CFU of *H. ducreyi* 35000, and the fourth subject received 10<sup>5</sup> CFU of live and heat-killed *H. ducreyi*. Lesions developed at all sites that received an inoculum of 10<sup>5</sup> CFU or greater and were mildly painful (Fig. 2). Both viable bacteria and abraded skin were required for lesion formation. Lesions also developed in two subjects who were rechallenged 2 months after the initial in-



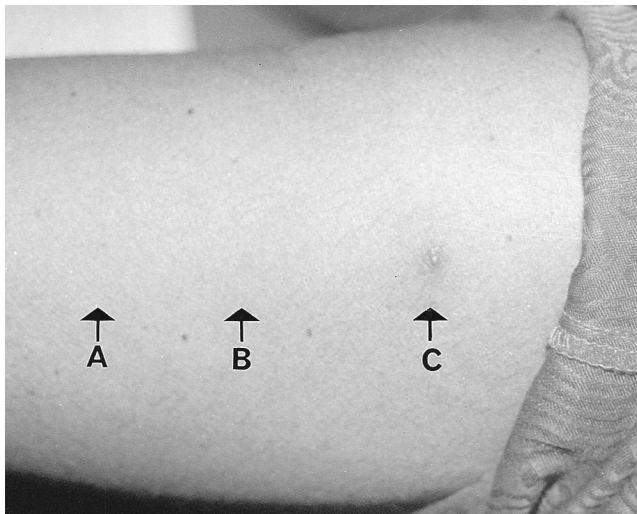


FIG. 2. Arm of volunteer from human challenge study (151), 3 days following inoculation with (A) PBS, (B)  $10^3$  heat-killed *H. ducreyi*, and (C)  $10^5$  live *H. ducreyi* delivered with an allergy testing device. Photo courtesy of S. M. Spinola, Indiana University, Indianapolis.

fection. Fever, lymphadenopathy, or dissemination of the infection did not develop in any of the subjects. Viable *H. ducreyi* cells were isolated from 10 of 12 biopsy specimens taken from the lesion area, although the number of bacteria recovered varied greatly. Histologic examination of lesion biopsy specimens revealed an infiltration of T cells, macrophages, and reactive endothelial cells. Western blotting revealed no evidence of a humoral immune response to either *H. ducreyi* proteins or LOS after primary or secondary infection. The use of a lower inoculation dose allowed patients to be infected for 2 weeks; however, the absence of a humoral immune response and the nature of the cellular infiltrate remained unchanged (147). This model should prove useful in studying the interactions of *H. ducreyi* and human skin cells during the initial stages of infection.

## POTENTIAL VIRULENCE FACTORS

### Pili

Until recently, it was thought that *H. ducreyi* did not possess pili. However, studies by Spinola et al. (148) have demonstrated that *H. ducreyi* does have pili, which appear as very fine surface appendages that are morphologically different from the pili found on *N. gonorrhoeae*. Pili were observed on all 12 *H. ducreyi* strains examined. Pilus expression was not readily lost during in vitro passage; however, pilus variation in vivo has yet to be studied. Pili have been purified from *H. ducreyi*, and after dissociation, the pilin monomer was found to have a molecular mass of 24 kDa in all strains examined (148). The pilin receptor on host cells has not been identified.

### Lipopolysaccharide

The lipopolysaccharide of *H. ducreyi* lacks the repeating O-antigens that are characteristic of enteric organism lipopolysaccharide. It is similar in size and structure to other mucosal pathogens, with a molecular mass of 3.5 to 6 kDa (91), and thus is more properly referred to as an LOS. Following intradermal injection, *H. ducreyi* LOS produces skin abscesses in rabbits, which are comparable in size to those produced by *N. gonor-*

*rhoeae* LOS and larger than those produced by *H. influenzae* or *E. coli* lipopolysaccharide (29). This tissue destruction suggests that *H. ducreyi* LOS may play a role in the pathogenesis of chancroid.

LOS may also play a role in the evasion of the host immune response by *H. ducreyi*. MAb 3F11 binds to *H. ducreyi* LOS on Western blots (95). The epitope recognized by MAb 3F11 is Gal1→4GlcNAc, which is also present in paragloboside, a glycosphingolipid precursor of the major human blood group antigen and of some gangliosides on human cells. This suggests that antigenic mimicry may enable *H. ducreyi* to evade certain host immune responses. The terminal Gal1→4GlcNAc of gonococcal LOS can be sialylated, converting a serum-sensitive organism to one that is serum resistant, providing the organism with an additional mechanism to counteract host defenses (92). It has recently been shown that *H. ducreyi* LOS is sialylated in a similar fashion (96).

Melaugh et al. (95, 96) determined that the LOS structure of *H. ducreyi* 35000 was Gal1→4GlcNAc1→4Gal1→4Hep1→6Glc1→(Hep1→Hep1→) 3,4Hep1→3-deoxy-D-manno-octulosonic acid (Kdo), with the reducing terminal Kdo existing in an anhydro form. More recent studies (96) indicated the presence of additional LOS glycoforms which resulted from the truncation or elongation of the major LOS form. Schweda et al. (142) determined the LOS structure from two additional strains of *H. ducreyi*. The LOS from strain ITM 2665 had a structure similar to that described for strain 35000. The structure of the LOS from strain ITM4747 was much simpler: Gal1→4Glc1→(Hep1→2Hep1→) 3,4Hep1→Kdo. This structure is very similar to that of the LOS from the nontypeable *H. influenzae* 2019. The proposed structures of *H. ducreyi* LOS are depicted in Fig. 3.

Recently, Campagnari et al. (27) obtained *H. ducreyi* LOS variants by selection for resistance to killing by *Pseudomonas aeruginosa* pyocins. One pyocin-resistant isolate produced a set of LOS molecules that were truncated in size compared with the LOS pattern of the wild-type strain. The LOS of this variant *H. ducreyi* strain no longer bound MAb 3F11 because of the loss of the terminal lactosamine, as demonstrated by immunologic and mass spectrometric techniques. LOS preparations from the variant strain also lacked sialic acid compared with LOS preparations from the wild-type strain, further supporting the hypothesis that the terminal lactosamine acts as a site for sialylation of *H. ducreyi* LOS (96).

### Iron-Regulated Proteins

A recent study by Lee (84) demonstrated the presence of four new proteins with masses of 40.5, 45.5, 50, and 65 kDa when *H. ducreyi* was grown under iron-limiting conditions. However, because of the heme requirement of *H. ducreyi*, these proteins may be upregulated by heme restriction instead of by iron restriction. Because of this heme requirement and the lack of any observable siderophores, Lee (84) proposed the presence of a heme receptor, which has yet to be identified. Another study (3) demonstrated that, when grown under conditions of iron limitation, *H. ducreyi* produced new proteins in the range of 43 to 160 kDa. The number and molecular masses of these new proteins varied among the four strains examined. Examination of electron micrographs revealed that organisms grown under conditions of iron limitation possessed an enlarged periplasmic space compared with bacteria grown under iron-replete conditions.

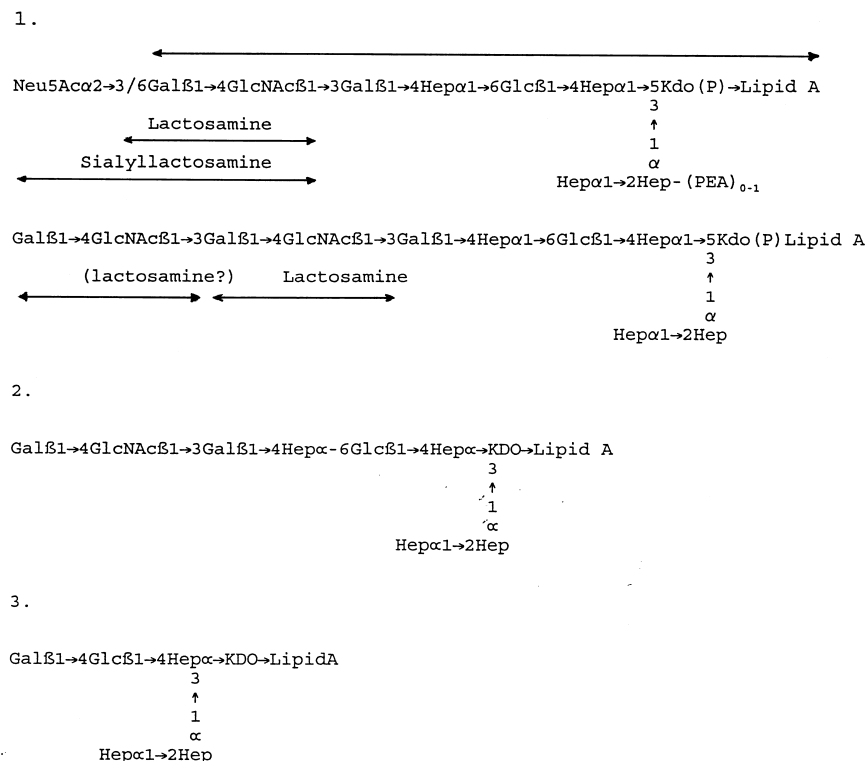


FIG. 3. Proposed structures of *H. ducreyi* LOS. 1, structure of LOS from strain 35000 (96); the line above the upper structure represents the major component of strain 35000 LOS. Minor components of 35000 LOS are shown in the lower structure, and variations of both structures are represented by lines below each structure. 2 and 3, structures of LOS from strains ITM2665 and ITM4747, respectively (142).

### Cytotoxins and Hemolysins

The question of whether *H. ducreyi* produces a cytotoxin(s) and the role that such a cytotoxin may play in chancre formation has been the subject of several studies (Table 7). Abeck et al. (2, 4) reported that filtrates from cultures of *H. ducreyi* did not contain extracellular enzymes, such as protease, elastase, lecithinase, lipase, collagenase, or IgA1 protease, which have been associated with virulence in other bacteria. In addition, culture filtrates had no cytotoxic activity against cultured Vero cells or human keratinocytes. Hollyer et al. (69) were also unable to detect the presence of extracellular *H. ducreyi* cytotoxins; CPE required either attachment or direct contact between *H. ducreyi* and the HFF cells. However, Purven and Langergard (122) observed cytotoxic activity against HEp-2 and HeLa cells. Cytotoxic activity was present in culture supernatants and preparations of sonicated or osmotically lysed cells. The cytotoxic effect was evident within 24 h and was heat and pronase sensitive. The toxic effect could be removed by incubating preparations with the target cells, which suggests that the putative cytotoxin was binding to the eukaryotic cells. No cytotoxic activity was seen against Vero or keratinocyte cell cultures. Rabbits immunized with *H. ducreyi* sonicates developed antibodies that neutralized the putative cytotoxin (81). Sonicates of *H. ducreyi* strains that did not produce the putative cytotoxin were unable to induce neutralizing antibodies. Preimmune sera as well as sera against other gram-negative bacteria were unable to neutralize the putative cytotoxin.

The production of hemolysins by *H. ducreyi* has been reported (84, 104). However, a recent study by Palmer et al. (111) was the first to address this issue in detail. Hemolytic activity is a property common to a number of *H. ducreyi* strains. The hemolysin lysed erythrocytes from various mammalian

sources; however, it was most active against horse erythrocytes and, to a lesser extent, against erythrocytes from rabbits, humans, and sheep. The hemolytic activity was sensitive to heat and protease treatment and was detected only in whole cells and not cell washes, sonicates, or supernatants. Thus, the hemolytic activity appeared to be distinct from the putative cytotoxin(s) described above. The hemolysin has not been purified, and its erythrocyte receptor or substrate has yet to be identified.

### Heat Shock Proteins

Heat shock proteins have been studied in a number of bacterial species and have been identified as a possible source of damage caused by cross-reactive antibodies and T cells (134). The heat shock operon of *H. ducreyi*, *groE*, was analyzed by Parsons et al. (114), who identified two adjacent open reading frames that exhibited a high degree of homology with the *groE* genes and GroES and GroEL proteins found in other bacteria. The two *H. ducreyi* genes *groES* and *groEL* encode predicted proteins of 10.3 and 57.8 kDa, respectively. Northern (RNA) blots demonstrated that growth of *H. ducreyi* at the optimum temperature (33°C) resulted in a high level of *groE* mRNA; heat shock, including the relatively small change from 33 to 37°C, provided a protective effect to *H. ducreyi* cells when they were rapidly chilled following growth at 33°C. Parsons et al. (114) reported that GroEL was the most predominant protein observed in *H. ducreyi* grown at 33°C in broth.

Brown et al. (24) identified proteins synthesized de novo during heat shock by using [<sup>35</sup>S]methionine. With this method, proteins of <14, 58.5, 74, and 78 kDa were concluded to be heat shock proteins. Western blot analysis with sera from rab-

bits immunized with *H. ducreyi*, sera from rabbits intradermally inoculated with *H. ducreyi*, pooled human serum from patients with chancroid, and a mouse MAb (BB11) was performed to determine whether heat shock proteins were immunogenic. Antibodies present in serum from infected rabbits and humans with chancroid and MAb BB11 reacted with the GroEL. Thus, GroEL appeared to be expressed *in vivo* and was immunogenic during infections.

### Outer Membrane Proteins

Few *H. ducreyi* outer membrane proteins (OMPs) have been characterized. Stewart et al. (152) cloned a 28-kDa protein that was common to all strains of *H. ducreyi* examined. Hiltke et al. (68) also cloned a gene from *H. ducreyi* that encoded a 28-kDa protein. This protein was conserved in all 30 strains examined and was shown by electron microscopy to be exposed on the surface. MAb 5C9, which binds to the 28-kDa protein, did not bind to several other gram-negative bacteria, suggesting that the epitope that this MAb binds may be specific to *H. ducreyi*. Whether this protein is the same as the 28-kDa protein described by Stewart et al. (152) remains to be determined.

Two additional *H. ducreyi* OMPs have been described by Spinola et al. (149, 150). An 18-kDa protein was observed in all strains of *H. ducreyi* examined. However, MAb 3B9, which was used to detect the 18-kDa protein, also bound to proteins of various sizes in other *Haemophilus* species. Sarkosyl insolubility and immunoelectron microscopy suggest that the 18-kDa protein was located in the outer membrane. The 18-kDa protein was not heat modifiable and did not exhibit phase variation during growth *in vitro*. A protein described as the major OMP (MOMP) of *H. ducreyi* had an apparent molecular mass of 39 to 42 kDa, depending on the strain examined (150). The *H. ducreyi* MOMP could be modified with heat, contained cysteine residues, and was cationic at a pH of 8.0, suggesting that it was not a classic porin protein (usually among the most abundant proteins in gram-negative bacteria). The N-terminal amino acid sequence and total amino acid content of the *H. ducreyi* MOMP exhibited homology to the OmpA proteins of members of the families *Enterobacteriaceae*, *Neisseriaceae*, and *Pasteurellaceae*. The function of this protein has yet to be determined; however, it may play a role in the serum resistance of this organism (125, 168).

### GENETICS AND CLASSIFICATION

Very little is known about the genetics of *H. ducreyi*. Therefore, the use of molecular biological techniques to study this organism has been limited. The construction of isogenic mutants of *H. ducreyi* will be necessary in order to evaluate the effect of a particular cellular component on pathogenesis. Two recent studies have explored the use of electroporation as a means of transforming *H. ducreyi*. Leong et al. (85) transformed plasmid DNA from clinical isolates of *H. ducreyi* into the reference strains ATCC 27722 and CIP542. Optimal electroporation conditions yielded a transformation frequency of approximately  $10^{-4}$ . Two beta-lactamase plasmids, pCH138 (7 MDa) and pCH137 (3.2 MDa), were used to transform the recipient strain to ampicillin resistance. The plasmids remained extrachromosomal and retained their original size following transformation. The transformation frequency was DNA concentration dependent up to a concentration of 0.75  $\mu\text{g/ml}$ . Above this concentration, only a slight increase in transformation frequency was seen. Electroporation was effective with both broth- and plate-grown cells.

Hansen et al. (65) obtained ca.  $10^6$  transformants per  $\mu\text{g}$  of

DNA when electroporation was used to transform *H. ducreyi* with the shuttle vector pLS88. Both linear and circular donor DNAs were successfully electroporated into the recipient cell, and transformation frequency was only slightly affected by DNA concentration. Electroporation has also been used to construct isogenic mutants of *H. ducreyi* (65). Two cloned *H. ducreyi* genes, which encoded surface-exposed proteins of 49 and 23 kDa, were inactivated by the insertion of antibiotic resistance cassettes and used to transform *H. ducreyi*. The transformation frequencies with these constructs were lower,  $10^1$  to  $10^3$  CFU/ $\mu\text{g}$  of DNA, than that obtained with pLS88; the transformants no longer reacted with MAbs that bind to the 49-kDa and 23-kDa proteins. Homologous recombination of the inactivated genes into the *H. ducreyi* chromosome was verified by Southern hybridization.

The ability to transform mutated genes, with successful homologous recombination, should prove valuable in the study of the virulence properties of specific *H. ducreyi* components in the pathogenesis of chancroid.

The issue of codon usage in *H. ducreyi* was evaluated by Parsons et al. (114). After analysis of the *groES* and *groEL* genes, it was determined that *H. ducreyi* used U- or A-rich codons 79% of the time. This figure is the same as that observed with *H. influenzae* and is much higher than the 45% reported for *E. coli*. As more genes from *H. ducreyi* are cloned, sequenced, and studied, additional information about the genetics of this organism will come to light.

The taxonomic position of *H. ducreyi* as a *Haemophilus* species has been questioned for a number of years. *H. ducreyi* was originally placed in the genus *Haemophilus* because of its requirement for hemin (X-factor) and a G+C content that was within the accepted range for *Haemophilus* spp. Albritton et al. (9) used genetic transformation, while Casin et al. (30) used DNA hybridization and S1 nuclease treatment to demonstrate that *H. ducreyi* was unrelated to true haemophili such as *H. influenzae*. De Ley et al. (44) used DNA-RNA hybridization to separate *H. ducreyi* from other haemophili but continued to classify *H. ducreyi* in the family *Pasteurellaceae*. Sequencing of the 16S rRNA of the type strain CIP542 (133) and two additional strains (48) confirmed that *H. ducreyi* was a member of the *Pasteurellaceae*. *H. ducreyi* has been assigned to cluster 4, while the true haemophili were assigned to cluster 1 (48). The groupings of the family *Pasteurellaceae* are very complex, and while *H. ducreyi* clusters differently from the true haemophili, renaming may have to wait until further investigation or reorganization of the entire family takes place.

### DEFINITION OF *H. DUCREYI* VIRULENCE

One area of importance to the study of chancroid pathogenesis is a definition of virulence for *H. ducreyi*. Various criteria to differentiate between so-called virulent and avirulent strains of *H. ducreyi* have been described. Resistance to polymyxin B was reported to correlate with virulence in a rabbit model for chancroid (63). However, when this association was reevaluated by using isogenic polymyxin B-resistant and polymyxin B-susceptible strains, there was no correlation between polymyxin B resistance and virulence (106). Resistance to the bactericidal action of 50% pooled normal human serum and resistance to phagocytosis and killing by human polymorphonuclear leukocytes have been used as criteria for virulence and to differentiate between strains that vary in their LOS composition (106). More recently, cell culture models have been used to differentiate between virulent and avirulent strains of *H. ducreyi* (11, 13, 82, 83). Perhaps the most widely accepted criterion for *H. ducreyi* virulence is the ability of a given strain



to produce a typical chancroidal lesion after intradermal inoculation into rabbits (55). By this criterion, the *H. ducreyi* type strain CIP542 has been designated avirulent. However, a recent laboratory infection with strain CIP542 (158) has focused attention on the definition of virulence. The course of this infection resembled that of chancroid. CIP542 has also been shown to be virulent in a mouse subcutaneous chamber model (159). Because of these observations, the definition of CIP542 as avirulent needs to be readdressed. In addition, the roles that antigenic and phase variation, colony variation, and extracellular products, such as hemolysins and cytotoxins, play in determining whether a strain of *H. ducreyi* is virulent or avirulent also deserve our increased attention.

#### AREAS FOR FUTURE CONSIDERATION

During the past 5 years, considerable progress has been made in the development of nonculture tests (i.e., PCR) and methods for strain identification. These methods should be used to address issues such as the prevalence of *H. ducreyi* in genital lesions, the role of asymptomatic carriers, and virulence differences among strains. PCR will also be useful in examining the etiology of genital ulcer disease in various geographic areas and in assessing the sensitivity of currently used diagnostic tests as well as the accuracy of a clinical diagnosis.

To further understand the pathogenesis of this interesting microorganism, additional studies are needed to identify and characterize the virulence factors of *H. ducreyi* and to elucidate the mechanism by which this microorganism causes the pathology associated with chancroid. A consensus is needed on a serological assay that can be used to assess the seroprevalence of chancroid in various populations. Standardized methods for performing agar dilution and disk diffusion antimicrobial susceptibility tests on *H. ducreyi* are needed. The propensity that *H. ducreyi* has shown for acquiring resistance plasmids from other microorganisms suggests the need for surveillance to ensure the efficacy of currently used treatment regimens. More studies are also needed on the physiology and metabolism of *H. ducreyi*; the results of such studies will be important in the development of better culture media. Finally, issues surrounding the taxonomic status of *H. ducreyi* should be resolved.

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