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Antimicrobial Susceptibility of *Haemophilus ducreyi*

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The susceptibility of 19 isolates of *Haemophilus ducreyi* from a recent chancroid outbreak and four reference strains was determined in vitro to 13 antimicrobial agents. The rabbit intradermal test for virulence was positive for all of the local isolates, but not for the reference strains. The "nonvirulent" reference strains were inhibited by lower minimum inhibitory concentrations (MICs) of most agents tested. For the virulent isolates, the range of MICs (in micrograms per milliliter) of the following were: of vancomycin, 8 to 128; of polymyxin, 32 to 128; of cloxacillin, 32 to 64; of tetracycline, 0.5 to 32; of cephalothin, 4 to 8; of doxycycline, 0.25 to 8; and of kanamycin, 1 to 8. Three strains were resistant to penicillin and ampicillin (MIC $\geq$ 128 μg/ml), and these three strains produced β-lactamase. The remainder were susceptible to 4 μg/ml. All strains were susceptible to rifampin (MIC $\leq$ 1 μg/ml), chloramphenicol (MIC $\leq$ 4 μg/ml), sulfisoxazole (MIC $\leq$ 8 μg/ml), and nalidixic acid (MIC $\leq$ 8 μg/ml). These susceptibilities of *H. ducreyi* indicate several antimicrobial agents that may be effective for chancroid treatment and support the use of vancomycin in a selective medium for the culture of chancroid genital ulcers.

A local outbreak of chancroid, an uncommon venereal disease, has occurred in Winnipeg, Canada, since July 1975. Nineteen isolates of *Haemophilus ducreyi* have been cultured from patients with this disease. Several studies have been reported previously on the in vitro susceptibility of the etiological agent *H. ducreyi* to a limited number of antimicrobial agents (1, 11, 12, 14, 16, 19, 20), with the most recent study in 1956. Conflict exists in the literature over reported in vitro susceptibility to penicillin (12, 14, 16, 19, 20). It is the purpose of this article to reevaluate the in vitro susceptibility of *H. ducreyi* and to consider which antimicrobial agents could be considered for therapy and as selective inhibitors in culture media.

Repeated laboratory passage of strains has been reported to alter virulence and antimicrobial susceptibilities (19). The virulence of the recent Winnipeg isolates and four reference strains was determined by the rabbit intradermal model at the time of antimicrobial susceptibility testing (4, 7).

MATERIALS AND METHODS

Bacterial strains. Eighteen strains of *H. ducreyi* were cultured from patients over the period July 1975 to February 1977. We have used the term strain to refer to an isolate from each different patient. An additional isolate was cultured by A. C. Maniar of the Cadham Provincial Laboratory of Manitoba from a genital sore. Four reference strains of *H. ducreyi* were obtained from the Pasteur Institute in Paris, strains CIP A75, A76, A77, and 54.2. Two reference gonococcal strains, F-13 and F-19, were obtained from the Center for Disease Control (CDC), Atlanta, Ga., courtesy of D. Kellogg. The reference strains *Staphylococcus aureus* ATCC 24923 and *Escherichia coli* ATCC 25992 were obtained from the American Type Culture Collection, Rockville, Md.

All organisms were small, pleomorphic gram-negative rods that grew in end-to-end chaining patterns in rabbit blood medium. They were identified grossly on agar by their nonmucoid, yellow-gray appearance and by the cohesive nature of colonies, which enabled them to be pushed intact across the agar surface. All strains required hemin (X factor) for growth independent of nicotinamide adenine dinucleotide (V factor), which was confirmed by a negative porphyrin test. Biochemically, all strains were oxidase, catalase, urease, and ornithine decarboxylase negative, did not produce indole or H_2S, and did not ferment glucose, sucrose, lactose, xylose, deoxyribose, or mannitol. One local and three reference strains did not reduce nitrate, the only variation from the identifying characteristics suggested by Kilian (9). Growth of all strains was enhanced by 5% CO_2 and high humidity.

Virulence testing. Virulence was tested by the intradermal (3, 5) injection of 0.15 ml of saline suspension containing approximately 10^9 colony-forming units (CFU) per ml into three, 2-kg, 1-year-old Australian white rabbits. Induration and necrosis were determined at 2, 4, and 11 days. The criteria for virulence were as follows: 0.5 cm of induration, with a central inflammatory response at 4 days that progressed to a central eschar by day 11. In three instances, the pustules were cultured at 72 h. The effect of repeated

subculture on virulence was examined by comparing the virulence of seven isolates after storage at −70°C refrigeration and after repeated in vitro subculture over a 6-month period (see Table 1).

Preparation of inocula. A 48-h culture of *H. ducreyi* grown on chocolate agar with 1% IsoVitaleX was scraped off the medium into Mueller-Hinton broth with 1% IsoVitaleX (IsoVitaleX was not included in the inocula or dilution media used for sulfonamide testing) and agitated for 3 s with a Vortex mixer. The thick suspension was allowed to sediment for 15 min, and the supernatant liquid was pipetted off and resuspended to an approximate dilution of 10^8 CFU/ml by turbidity standards (0.5 McFarlane). Cultures (24-h-old) of gonococcal strains were suspended in the broth to give an inoculum size approximating 10^8 CFU/ml. The inocula for *S. aureus* and *E. coli* were obtained by sampling from four to five colonies after overnight growth and inoculation into 2 ml of the supplemented Mueller-Hinton broth. After a 2-h incubation, the approximately 10^8 CFU/ml was diluted to 10^6 CFU/ml. These suspensions of microorganisms were inoculated onto the surface of the agar with a Steers-Foltz replicator (17).

Antimicrobial agents. The bacteria were tested for susceptibility to rifampin (Dow Chemical Co., Indianapolis, Ind.); chloramphenicol (Parke, Davis & Co., Detroit, Mich); doxycycline (Pfizer Inc., New York); tetracycline, kanamycin, ampicillin, penicillin, and cloxacillin (Bristol Laboratories, Syracuse, N.Y.); naladixic acid (Winthrop Laboratories, Aurora, Ontario); sulfisoxazole (Hoffman-LaRoche, Inc., Nutley, N.J.); cephalothin (Eli Lilly & Co., Indianapolis, Ind.); vancomycin (Eli Lilly & Co., Toronto, Ontario); and polymyxin B (Sigma Chemical Co., St. Louis, Mo.). Stock solutions were prepared in appropriate diluents, and further dilutions were made in agar.

Agar dilution technique. The agar dilution technique (3, 15) was carried out in duplicate on gonococcal medium base (Difco Laboratories, Detroit, Mich.) enriched with 1 g of hemoglobin (Difco) per 100 ml and 1% IsoVitaleX (Baltimore Biological Laboratory, Cockeysville, Md.). Rifampin, chloramphenicol, cephalothin, doxycycline, tetracycline, nalidixic acid, kanamycin, cloxacillin, ampicillin, penicillin, polymyxin, and vancomycin were incorporated into the agar at 50°C to yield final concentrations increasing in a $\log_2$ dilution series from 0.005 to 128 μg/ml. Sulfisoxazole was incorporated into gonococcal base enriched with 0.1% glucose, 0.01% glutamine, 0.025% hemin (Sigma Chemical Co.) (5) and 5% lysed horse blood. The agar was poured into 90-mm petri dishes to a depth of 4 mm and was used within 1 week of preparation after storage at 5°C. The penicillins and cephalothin were used within 24 h of preparation. The inoculated plates were incubated with 5 to 10% CO_2 at 33°C in an atmosphere saturated with water vapor. The plates were read independently after 24 and 48 h by two observers. If a difference in reading occurred, the lower of the two minimum inhibitory concentrations (MICs) was taken as the true value. The MIC of each antimicrobial agent was considered to be the lowest concentration that allowed growth of three colonies or less or that resulted in a haze that did not appear to be elevated above the surface of the agar when a hand-held lens (×5) was used. The MIC for sulfisoxazole was taken as the lowest concentration that inhibited 80% or more of the growth in comparison with the sulfonamide-free control plate. All controls were read at 24 h, and values for each agent were reported only if the controls were within one MIC dilution of the known value. Due to the slow growth of the organisms, the MICs reported for *H. ducreyi* were read at 48 h.

Penicillinase production. The presence of β-lactamase was determined by the chromogenic cephalosporin test (13). A heavy inoculum of each bacterial isolate was scraped from the surface of the chocolate agar plate and suspended in microtiter wells containing 0.05 ml of sterile normal saline, to which 0.05 ml of the chromogenic cephalosporin was added. Positive controls were strains of β-lactamase producing *Haemophilus influenzae* and *Neisseria gonorrhoeae* CDC 76-073389. Negative controls consisted of a well without bacteria and a known non-β-lactamase-producing *H. influenzae*.

RESULTS

All 19 Winnipeg strains were virulent by the criteria outlined above, whereas the four reference strains were nonvirulent (Table 1 and Fig. 1). Seven Winnipeg isolates after 63 subcultures, each over 6 months, were still virulent. The pustules produced by three Winnipeg strains that were sampled 72 h after inoculation grew *H. ducreyi* in pure culture.

The antimicrobial susceptibilities of the 23 strains are listed in Table 2. The avirulent strains were more susceptible to all of the antimicrobial agents tested. *H. ducreyi* was relatively resistant to cloxacillin, vancomycin, and polymyxin, with MICs $\geq$ 32 μg/ml for all but one virulent organism. The isolates were relatively susceptible to rifampin, doxycycline, sulfisoxazole, nalidixic acid, cephalothin, kanamycin, and chloramphenicol. However, three isolates (54198, 54207, 54211) were resistant to penicillin and ampicillin, and one of these (54198) was also resistant to tetracycline. The test for β-lactamase was positive within 5 min for these three isolates of *H. ducreyi*.

DISCUSSION

Previous investigators have used a limited number of strains of *H. ducreyi* (1 to 16 isolates) to evaluate antimicrobial susceptibility. Although in vitro susceptibility of *H. ducreyi* has been found to be related to virulence (16), virulence was not determined in some studies (1, 20), and in another study predominantly avirulent strains were tested (19). Techniques of susceptibility testing have varied widely.

In the present study, all four of the older reference strains obtained from the Pasteur Institute in Paris were nonvirulent by our criteria and were much more susceptible to most antimicrobial agents than were the virulent Winni-

TABLE 1. *Results of virulence determination of H. ducreyi (intradermal inoculation in rabbits)*

Strains	Strain no.	Induration (cm) at:		Central reaction at:	
		2 days	4 days	4 days[a]	11 days[b]
Winnipeg on initial cultures (after two transfers)	35000	1.4	1.1	4+	+
	36652	1.8	1.3	4+	+
	78118	1.1	0.7	1+	+
	78226	1.5	0.9	2+	+
	82038	1.8	1.4	4+	+
	54182	1.8	1.0	3+	+
	54183	1.4	0.9	2+	+
	35001	1.2	1.0	3+	+
	35199	1.3	0.8	3+	+
	54198	1.4	1.1	4+	+
	54201	1.3	1.1	3+	+
	54202	1.3	0.6	2+	+
	54204	1.1	0.6	2+	+
	54205	1.1	1.0	3+	+
	54207	1.7	1.3	4+	+
	54209	1.8	1.2	4+	+
Winnipeg after multiple (63) transfers	35000	1.5	1.1	4+	+
	36652	2.0	1.2	4+	+
	78118	1.4	1.0	1+	+
	78226	1.4	1.1	2+	+
	82038	1.7	1.2	4+	+
	54182	1.5	1.2	4+	+
	54183	1.8	1.1	2+	+
Reference	CIP 54.2	1.1	0	1+	0
	CIP A75	1.0	0	0	0
	CIP A76	0.5	0	0	0
	CIP A77	0.4	0	0	0

[a] Central reaction based on color change at 4 days: 4+, black central inflammatory response; 3+, brown central inflammatory response; 2+, red central inflammatory response; 1+, pink central inflammatory response.
[b] Central reaction based on the presence of hard central eschar at 11 days: +, eschar present; 0, no eschar.

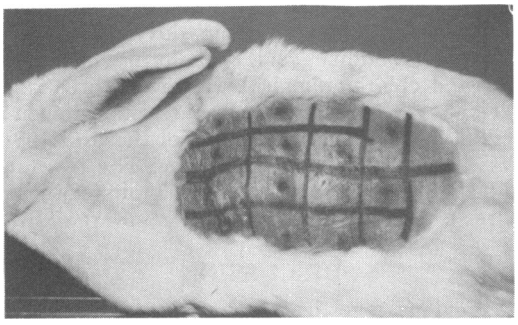

FIG. 1. *Virulence of seven strains of H. ducreyi (3500 to 54183, Table 1) from left to right, with top two rows after 63 passages versus 2 passages in vitro for rows 3 and 4, 4 days after rabbit intradermal injection, showing no change in virulence for each corresponding strain.*

peg strains. However, all of the latter strains were from one localized outbreak of chancroid. Attempts to make our Winnipeg isolates avirulent by repeated passage has not been successful to date. Thayer succeeded in making a strain avirulent after 44 passages in vitro, without altering its susceptibility to polymyxin (19). However, his six avirulent strains were much more susceptible to polymyxin than was his initial virulent strain. No hypothesis has satisfactorily accounted for both the change in susceptibility and the loss of virulence in *H. ducreyi*.

These susceptibilities support previous laboratory and clinical observations on disease caused by *H. ducreyi*. This organism has been shown to be susceptible to tetracycline (19), streptomycin (14, 19), chloramphenicol (19), sulfonamides (11, 14), and penicillin (12, 14, 16, 19) in vitro. In our experience, 1 g of sulfisoxazole administered orally four times daily for 7 to 14 days or 500 μg of tetracycline administered orally four times daily for 7 to 14 days has been an effective treatment for chancroid. In two of our patients not initially diagnosed as chancroid, oral cloxacillin proved ineffective, whereas four patients were cured with 1 week of oral or parenteral penicillin. Penicillin has proven to be an

TABLE 2. *Susceptibility of 23 strains of H. ducreyi to 13 antimicrobial agents*

Strain	Susceptibility (μg/ml)												
	Rifampin	Chloram-phenicol	Sulfisoxa-zole	Cephalo-thin	Doxycycline	Tetracy-cline	Nalidixic acid	Kanamycin	Cloxicillin	Ampicillin	Penicillin	Polymyxin	Vancomycin
35000	0.016	1	2	4	0.25	1	1	2	64	1	1	>128	128
35001	0.016	0.5	0.5	4	0.25	0.5	1	8	64	1	1	>128	64
35199	0.063	0.5	0.5	4	0.25	1	1	4	64	1	1	>128	128
36652	0.031	0.5	2	4	0.5	1	8	8	64	1	1	>128	64
78118	0.016	0.25	2	4	0.5	1	1	4	64	1	1	>128	64
78226	0.016	0.5	2	4	0.5	2	2	4	64	1	1	>128	32
82038	0.016	0.5	1	4	0.5	1	1	4	64	1	1	>128	64
54182	0.016	0.5	4	4	0.5	0.5	1	2	64	1	1	>128	64
54183	0.016	0.5	8	4	0.5	0.5	1	4	64	1	1	>128	32
54189	0.016	0.13	0.5	4	0.5	0.5	1	4	64	1	1	>128	128
54198[a]	0.016	4	0.13	8	8	32	8	1	32	>128	>128	>128	8
54201	0.16	0.25	8	4	0.5	1	1	8	64	1	1	>128	64
54202	0.031	0.5	NT[b]	4	1	1	1	4	64	2	1	>128	64
54204	1	0.5	4	4	0.5	0.5	1	4	64	2	4	>128	64
54205	0.016	0.5	4	4	0.5	0.5	1	4	64	1	1	>128	32
54207[a]	1	0.13	0.5	8	0.13	0.5	4	1	32	>128	>128	32	128
54209	0.031	0.25	0.5	4	0.25	0.5	1	8	64	2	1	>128	32
54211[a]	NT	NT	0.13	4	1	NT	NT	NT	32	128	128	NT	NT
54213	NT	NT	2	4	0.5	NT	NT	2	64	1	1	NT	NT
CIP A75	0.016	0.13	NT	0.25	0.13	0.13	2	NT	2	0.13	0.13	0.5	16
CIP A76	0.016	0.5	NT	0.25	0.13	0.25	4	NT	4	0.13	0.13	0.5	16
CIP A77	0.031	0.25	0.5	0.13	0.25	0.25	4	NT	2	0.13	0.13	0.5	32
CIP 54.2	0.16	0.25	1	0.13	0.25	0.5	2	NT	1	0.13	0.13	4	4
GC007	0.5	0.5	1	0.25	0.5	1	1	16	0.5	0.13	0.063	>128	32
GC009	4	1	8	8	2	2	1	32	64	1	2	>128	>128
E. coli	64	8	64	8	4	2.0	4	32	>128	4	0.125	0.5	>128
S. aureus	0.031	8	64	0.06	0.25	0.5	32	2	0.25	0.13	32	>128	2

[a] Positive for β-lactamase production.
[b] NT, not tested.

effective treatment for chancroid of the cervix (2), when coexisting with primary syphilis (18) and in volunteers inoculated with *H. ducreyi* (14). However, the emergence during a localized chancroid outbreak of penicillin-resistant, β-lactamase-producing *H. ducreyi* is ominous. Further studies are underway to characterize the β-lactamase of these three isolates to determine whether it is plasmid mediated. One of these three isolates was also resistant to tetracycline and doxycycline. Resistance to tetracycline has been observed in Viet Nam in patients with chancroid (8, 10). Our strains were relatively susceptible to kanamycin and cephalothin, the antimicrobial agents used to treat patients with resistant infections (6, 10).

The level of in vitro resistance to vancomycin and polymyxin is comparable to that of most *Neisseria* species. A selective culture medium containing 3 μg of vancomycin per ml of chocolate agar enriched with 1% IsoVitaleX has proven effective in a prospective study of the isolation of *H. ducreyi* from chancroid genital ulcers (5).

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