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Antimicrobial Susceptibility and Characterization of Outer Membrane Proteins of *Haemophilus ducreyi* Isolated in Thailand

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One hundred strains of *Haemophilus ducreyi* isolated in Thailand from patients with chancroid were tested by the agar dilution method against 10 antimicrobial agents and typed by outer membrane protein pattern by using sodium dodecyl sulfate gel electrophoresis. All strains produced beta-lactamase and were resistant to tetracycline, kanamycin, and sulfonamides. Most had a decreased susceptibility to trimethoprim (MIC for 50% of the strains [MIC_{50}], 0.5 μg/ml) and chloramphenicol (MIC_{50}, 8 μg/ml). Strains were susceptible to ciprofloxacin (MIC_{90}, 0.001 μg/ml), ceftriaxone (MIC_{50}, 0.0015 μg/ml), erythromycin (MIC_{50}, 0.015 μg/ml), rosoxacin (MIC_{50}, 0.03 μg/ml), and spectinomycin (MIC_{50}, 8 μg/ml). The degree of antimicrobial resistance found in Thailand is higher than that reported for *H. ducreyi* isolated in other regions. Five different outer membrane protein patterns were found by analyzing proteins in the range of 29 to 61 kilodaltons, but 98% of the Thai strains fell into three patterns which did not differ greatly. Outer membrane protein patterns of Thai strains were also seen in strains from other geographic areas. A new outer membrane protein type was found among nine strains isolated in Singapore.

Haemophilus ducreyi is the cause of chancroid, a sexually acquired disease characterized by genital ulcers and tender inguinal lymphadenopathy. Selective primary isolation media have made the routine isolation of this organism from clinical specimens possible (7, 22) and have stimulated considerable interest in the clinical, epidemiological, and bacteriological aspects of chancroid. Chancroid is a common venereal disease in Thailand (24) and many other countries in the tropics (4, 13, 19). Outbreaks in the United States and Canada have demonstrated that *H. ducreyi* can be acquired and transmitted in temperate climates as well (3, 9). Although a variety of antimicrobial agents have been used for the treatment of chancroid, little is known about the susceptibility of Asian strains to the recommended drugs (11). Effective methods of control of chancroid depend on understanding the epidemiology of chancroid (18). In this study *H. ducreyi* strains isolated in Thailand were characterized by antimicrobial susceptibility and outer membrane protein (OMP) patterns to identify antimicrobial agents for treatment and as a means to type strains.

MATERIALS AND METHODS

Strains. One hundred strains of *H. ducreyi* were isolated in 1982 and 1983 at Bangrak and Royal Thai Army hospitals located in Bangkok. All strains were isolated from genital ulcers, and all except two were isolated from males. Two media were used for primary isolation of *H. ducreyi*: one consisted of Mueller-Hinton agar base (Difco Laboratories) containing 5% chocolatized horse blood, 5% fetal calf serum, 1% IsoVitaleX (BBL Microbiology Systems), and 3 μg of vancomycin per ml (4); and another consisted of heart infusion agar base (GIBCO Laboratories) containing 5% rabbit blood, 5% fetal calf serum, and 3 μg of vancomycin per ml (22). Colonies with characteristic morphology were confirmed as *H. ducreyi* by standard methods (7). A heavy inoculum of each strain was stored in skim milk at −70°C and later transported on dry ice to P. Piot in Belgium and A. Ronald in Canada for antimicrobial susceptibility testing.

The following *H. ducreyi* strains were used for comparison of OMP patterns. Strain 36-F-2 (originally from Copenhagen) was provided by W. Albritton. Strains V1158 and V1159 (isolated in Seattle from patients infected in the Philippines), 109 and Johnson (Atlanta), 4391 (Amsterdam), HD1 (Sweden), 54189 and 54198 (Winnipeg), and 9468 and 9926 (Nairobi) were provided by A. Ronald. Strains 4, 5, 6, 27, 30, 31, 43, 72, and 93 isolated in Singapore were provided by E. H. Sng.

Antimicrobial susceptibility testing. Isolates were examined for beta-lactamase by the chromogenic cephalosporin test (14). A total of 88 *H. ducreyi* strains were tested in Antwerp with the following antimicrobial agents: erythromycin, tetracycline, and kanamycin obtained from the Ministry of Health, Brussels; spectinomycin (The Upjohn Co.); ceftriaxone (Rocephin RO 13-9904/01; F. Hoffman-La Roche & Co.); rosoxacin (Win 35213; Winthrop Laboratories); and chloramphenicol (Zambon). Inocula were prepared in broth medium containing Mueller-Hinton broth (BBL), 200 mg of hemin hydrochloride per ml (BDH), 5% horse serum (GIBCO), and 2% IsoVitaleX (BBL). Twofold dilutions of the antibiotic compounds were incorporated in test plates containing Mueller-Hinton agar (BBL), 5% chocolatized horse blood (GIBCO), 5% calf serum (GIBCO), and 2% IsoVitaleX (BBL) (8). Overnight broth cultures were used to inoculate these plates with a Steers Multipoint inoculator (Dembey Tech, Ltd.), giving a final inoculum of 10^5 CFU/ml. Clumping of *H. ducreyi* was reduced by shaking the broth cultures rigorously for 30 s on a vortex mixer. The test plates were incubated for 48 h in 5% CO_2 at 35°C. Susceptibilities to ciprofloxacin (Bayer), trimethoprim, and sulfamethoxazole were tested for 85 *H. ducreyi* strains in Canada by using similar methods, except twofold dilutions of trimethoprim

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TABLE 1. MICs of 10 antibiotics for *H. ducreyi* strains isolated in Thailand, 1982–1983

Antibiotic	No. of strains	MIC (μg/ml)[a]		
		50%	90%	Range
Ciprofloxacin	85	0.001	0.001	<0.0005–0.001
Ceftriaxone	75	0.0015	0.003	0.0007–0.007
Erythromycin	70	0.015	0.03	0.007–0.06
Rosoxacin	77	0.03	0.06	0.03–0.06
Trimethoprim	85	0.5	4.0	0.12–>16
Chloramphenicol	69	8.0	16.0	4–16
Spectinomycin	75	8.0	8.0	4–16
Tetracycline	72	>32	>32.0	>32
Kanamycin	75	>250	>250	>250
Sulfamethoxazole	85	>160	>160	>160

[a] 50% and 90%, MICs for 50 and 90% of the isolates, respectively.

and sulfamethoxazole were incorporated into test plates containing Mueller-Hinton agar, 5% lysed horse blood (GIBCO), 0.1% glucose, 0.01% glutamine, and 0.025% cysteine (6).

Isolation of OMPs. Detergent-insoluble outer membranes of *H. ducreyi* were prepared as previously described with the following modifications (15). Samples (50 μl) were separated in 1.5-mm-thick 12% bisacrylamide–sodium dodecyl sulfate gel slabs in an electrophoresis chamber (Hoefer Scientific Instruments). Electrophoresis was carried out at a constant current of 60 mA for 4 h and 45 min. Gels were fixed and stained overnight in a solution of 0.05% Coomassie blue–50% methanol–10% (wt/vol) trichloracetic acid and restained in a solution of 7.5% acetic acid.

The molecular weight standards (Bio-Rad Laboratories) used were phosphorylase *b* (92,500), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

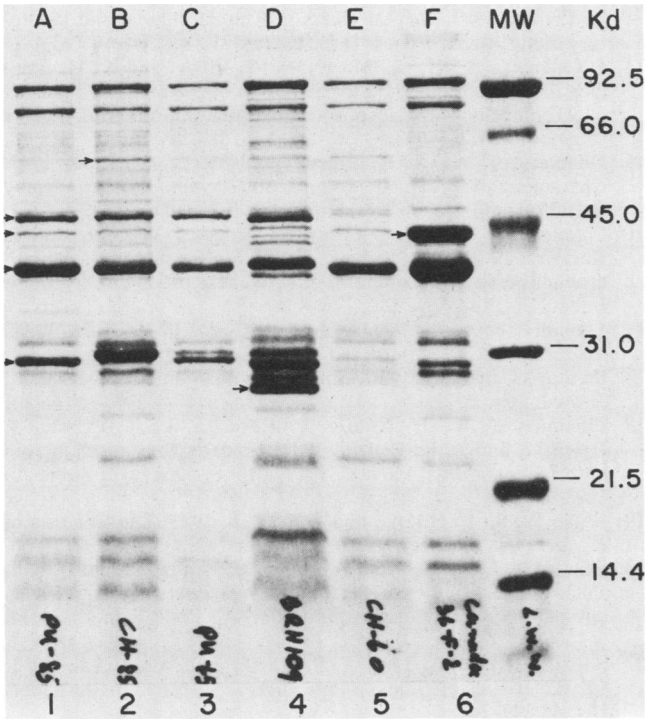

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of sodium lauryl sarcosinate-insoluble OMPs of *H. ducreyi* types. Lanes A through E contain strain types isolated in Thailand; lane F contains reference strain 36-F-2; MW refers to protein standards ranging in molecular mass from 92.5 to 14.4 kd. Arrows indicate proteins that characterize strain types (see text and Table 2). Types are indicated below each gel lane. Lane A, strain PU-85 (type 1); lane B, strain CH-85 (type 2); lane C, strain PU-65 (type 3); lane D, BRH-109 (type 4); lane E, CH-60 (type 5); lane F, 36-F-2 (type 6).

RESULTS

Antimicrobial susceptibility. All strains produced beta-lactamase and were resistant to tetracycline, kanamycin, and sulfonamides. The results of antimicrobial susceptibility testing are seen in Table 1.

OMP patterns. The OMP patterns are shown in Fig. 1 and Table 2. The OMP profiles of *H. ducreyi* strains were reproducible from one gel preparation to the next. OMP patterns of strains isolated from multiple ulcers from the same patient also were identical. In Fig. 1, lanes A through E represent the patterns of strains isolated in Thailand, and lane F represents the pattern of strain 36-F-2. All *H. ducreyi* strains examined have a major OMP with a molecular mass of 39.4 kilodaltons (kd). Lane A has prominent OMPs with molecular masses of 47.4, 44.2, 39.4, and 30.5 kd (arrows), and that strain is designated as type 1. Lane B (type 2) contains a strain which is similar to the type 1 strain but has an OMP of 56.9 kd (arrow) and a less prominent OMP of 44 kd. Lane C (type 3) has all bands found in both types 1 and 2. Lane D (type 4) is similar to type 2 (lane B) but has an additional OMP of 26.9 kd (arrow). Lane E (type 5) lacks prominent bands at 47.3 and 30.5 kd. Lane F (type 6) shows very prominent OMP bands at 44.2 kd (arrow) and 39.4 kd. The type 6 pattern is not seen in Thailand. By using this typing system to type strains isolated outside of Thailand, strains V1158, V1159, 109, 4391, HD1, 9926, and eight of nine strains from Singapore were type 1, strain 9468 was type 4, strain Johnson was type 5, and strain 54189 was type 6. Singapore strain 4 was similar to type 1 but had a unique OMP band at 40.7 kd and was designated as type 7 (data not shown).

DISCUSSION

High-degree kanamycin resistance has not been reported for *H. ducreyi* strains isolated in Africa (20), but it has been reported in 11 of 19 strains isolated in Amsterdam, presumably from travelers (23). Tetracycline resistance (MIC, ≥8 μg/ml) has now been reported in more than 50% of the strains isolated in the Philippines, Singapore; Nairobi, Kenya; and Johannesburg, South Africa (1, 5, 10, 21).

All Thai strains are resistant to sulfamethoxazole (MIC, >64 μg/ml). Resistance has been reported in about one-third of the strains in Nairobi (range, 16 to 56%) and in 10% of the strains in Johannesburg (1, 5, 6, 17). Resistance to trimethoprim (MIC, >2 μg/ml) has not been reported in Nairobi but

TABLE 2. Characterization of *H. ducreyi* by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of composition

Type	Molecular masses of major OMPs (kd)	No. of strains
1	47.3, 44.2, 39.4, and 30.5	15
2	56.9, 47.3, 39.4, and 30.5	25
3	56.9, 47.3, 44.2, 39.4, and 30.5	58
4	56.9, 47.3, 39.4, 30.5, and 26.9	1
5	39.4	1
6	44.2 and 39.4	2[a]
7	47.3, 40.7, 39.4, and 30.5	1[a]

[a] Patterns not present in strains isolated in Thailand.

is found in 10% of the isolates from Thailand and in 50% of the isolates from Johannesburg (1, 5, 6, 17). The combination trimethoprim-sulfamethoxazole has been reported as 100% effective treatment for chancroid in Africa, probably because *H. ducreyi* isolates are susceptible to trimethoprim in Nairobi and to sulfamethoxazole in Johannesburg (1, 5, 6, 17). Since Thai strains are uniformly resistant to sulfamethoxazole treatment, failures would be expected in patients infected with trimethoprim-resistant strains.

H. ducreyi strains from Johannesburg were more susceptible to chloramphenicol (MIC for 50% of the strains, 0.25 μg/ml) (1), and thiamphenicol has been found to be effective therapy (12). However, resistance reported from Bangkok, the Philippines (10), and Amsterdam (23) suggests that chloramphenicol or thiamphenicol would be poor treatment choices for strains isolated outside of Africa.

Strains of *H. ducreyi* isolated in Thailand are very susceptible to ciprofloxacin, ceftriaxone, erythromycin, and rosoxacin. Erythromycin has been effective treatment in Korea, Nairobi, and Johannesburg (2, 4, 16). Rosoxacin was as effective as erythromycin in Nairobi (16). In vitro susceptibilities suggest that ceftriaxone and ciprofloxacin also would be effective.

The OMP patterns of *H. ducreyi* have been examined only once previously (15). The common *H. ducreyi* OMP was described as having a molecular mass of 40 kd, very similar to the 39.4-kd OMP reported in this study. The OMP patterns of strains 36-F-2 and V1159 were examined in both studies and provide a useful comparison. Strain 36-F-2 appears the same in both studies, with large OMPs of approximately 40 and 45 kd. However, strain V1159 (type 1) was described as having a prominent OMP of 38 kd instead of 31 kd, as reported in the present study. In general, there appears to be agreement with OMPs of 39 kd and larger, but not with OMPs smaller than the common OMP of 39.4 kd. The lower acrylamide gel concentration and the higher electrophoresis current used in the present study may have obtained better separation of the low-molecular-weight OMPs.

Among the Thai strains five different OMP patterns were found. Of the strains, 98% could be categorized into three patterns which differed by the relative size of the 44.2- and 56.9-kd OMP bands. These differences are small and at times not clear-cut. The differences between these types and types 4, 5, 6, and 7 are much more clearly defined, but types 4 through 7 unfortunately are rare in Thailand. The OMP profiles of *H. ducreyi* strains isolated in Thailand and Singapore were very similar. The type 1 pattern also was seen in both strains acquired in the Philippines and in the majority of the 72 strains isolated in Nairobi (15). Because of these similarities, OMP profiles appear to have limited usefulness as a method to type strains of *H. ducreyi*.

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