JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1991, p. 1949–1954
0095-1137/91/091949-06$02.00/0

Vol. 29, No. 9

Molecular Characterization of *Haemophilus ducreyi* by Ribosomal DNA Fingerprinting

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Intraspecies genotypic heterogeneity among *Haemophilus ducreyi* isolates was examined by using genomic fingerprints with rRNA from *Escherichia coli* as a probe. DNA from 44 isolates of *H. ducreyi* was digested by restriction endonuclease *Hin*cII or *Hin*dIII, separated by agarose gel electrophoresis, transferred to nylon membranes, and hybridized with ^{32}P-labeled 16S and 23S rRNA. *Hin*cII digests yielded four hybridization patterns (ribotypes), whereas *Hin*dIII digests yielded eight ribotypes. Four *Hin*cII and five *Hin*dIII ribotypes were observed among 14 *H. ducreyi* isolates collected within a period of 1 month in Kenya, where chancroid is endemic. In contrast, one *Hin*cII and two *Hin*dIII ribotypes were observed among 28 isolates collected during the Orange County, Calif., chancroid epidemic that occurred in 1981 and 1982. The plasmid content, in conjunction with ribotyping, provided additional differentiation among some isolates of *H. ducreyi*. This study demonstrates that ribotyping of *H. ducreyi* may be used to study the epidemiology of chancroid.

Chancroid is a sexually transmitted disease characterized by cutaneous erosion or ulceration. The true incidence of chancroid is unknown because of insensitive culture methods and reliance on a clinical diagnosis based on the appearance of the ulcer and response to empirical therapy (6, 21). Although chancroid is considered uncommon in the United States, more than 5,000 cases were reported in 1987 (12), more than a sixfold increase since 1984. The etiological agent of chancroid is *Haemophilus ducreyi*, a small, gram-negative, nonmotile rod that is relatively biochemically inactive (12). The epidemiology of chancroid is poorly understood because of the lack of typing methods that would permit differentiation among strains of *H. ducreyi*. Strains of *H. ducreyi* have been characterized phenotypically by outer membrane protein profiles (13, 22), indirect immunofluorescence (18), enzyme profiles (24), and lectin typing (10). Although these methods have provided some differentiation among isolates, they lack the power to differentiate extensively among many isolates. Moreover, the phenotypes detected by these methods may not be stable.

A recent development in DNA analysis, ribotyping, is based on restriction fragment length polymorphisms of rRNA genes. Several investigators (7, 20) have demonstrated that the hybridization of restriction endonuclease-digested chromosomal DNA with rRNA from *Escherichia coli* (ribotyping) could be used to identify bacterial species or strains (15) and to investigate the molecular epidemiology of genetically diverse bacteria (8, 25). Ribotyping has also been used to differentiate among strains of *Salmonella typhi*, a species once thought to be a single clone based on the results of other typing systems (2). In this study, we determined the potential utility of ribotyping for differentiating among strains of *H. ducreyi*.

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MATERIALS AND METHODS

Strains. Forty-four clinical isolates of *H. ducreyi* were included in this study (Tables 1 and 2). Thirteen of these isolates were initially examined; they were collected between 1981 and 1990 in different geographic areas and included three pairs of isolates from each of three geographic locations. Eighteen isolates of *H. ducreyi* were obtained from a chancroid epidemic that occurred in Orange County, Calif., in 1981 and 1982 (4); these represented approximately 20% of the positive cultures over a 5-month period during the epidemic. Fourteen isolates were from Nairobi, Kenya, and were collected during a 1-month period in 1984. *H. ducreyi* isolates were grown on heart infusion agar base (Becton Dickinson, Cockeysville, Md.) supplemented with 5% (vol/vol) defibrinated rabbit blood, 1% (vol/vol) IsoVitaleX (Becton Dickinson), and 5% (vol/vol) fetal bovine serum.

Plasmid content. The plasmid profiles of *H. ducreyi* isolates were determined as described previously (11). The 3.2-MDa plasmid present in the Orange County, Calif., isolates encoded β-lactamase and was previously characterized (3).

DNA preparation. DNA was prepared by a modification of the method of Brenner et al. (5). Strains were grown on chocolate agar (GC medium base; Becton Dickinson) containing 1% (vol/vol) IsoVitaleX, 1% (wt/vol) hemoglobin, and 10% (vol/vol) fetal bovine serum in a candle jar incubated at 33°C for approximately 48 h. Cells were removed from the agar plate by suspension in 1.5 ml of 1× SSC (20× stock contained 175.3 g of NaCl and 88.2 g of sodium citrate per liter [pH 7.0]). A 0.5-ml aliquot of this suspension was deposited in an Eppendorf microcentrifuge tube, and the bacteria were sedimented by centrifugation at 15,600 × *g* for 1 min. Cells were then washed in 0.5 ml of STE (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA), sedimented by centrifugation, and suspended in 0.5 ml of STE. After the addition of 30 μl of 10% (wt/vol) sodium dodecyl sulfate (SDS) and 15 μl of RNase A (2 mg/ml; Boehringer Mannheim, Indianapolis, Ind.), the tubes were incubated at 37°C for 30 min; then 3 μl of proteinase K (20 mg/ml; Boehringer Mannheim) was added, and incubation at 37°C was contin-

TABLE 1. Geographic source, *Hin*cII and *Hin*dIII ribotypes, and plasmid content of selected isolates of *H. ducreyi*

Isolate[a]	Year of isolation	Geographic source	Ribotype		Plasmid size(s) (MDa)
			*Hin*cII	*Hin*dIII	
ATCC 33940 (CIP542)	1954	Hanoi, Viet Nam	1	1	No plasmid
ATCC 33922	1975	Winnipeg, Canada	1	1	No plasmid
V-1159	1979	Seattle, Wash.	1	2	7.0
C148		Kenya, Africa	1	3	No plasmid
HD-187	1982	Kenya, Africa	1	2	7.0
HD-188	1982	Kenya, Africa	1	2	7.0
HD-181	1982	Atlanta, Ga.	2	4	4.5, 5.7
HD-182	1982	Atlanta, Ga.	2	4	4.5, 5.7
HD-179	1982	Orange County, Calif.	2	5	3.2
HD-185	1984	West Palm Beach, Fla.	1	2	5.7
HD-186	1984	West Palm Beach, Fla.	1	2	5.7
HD-174	1989	Tampa, Fla.	2	4	No plasmid
HD-189	1990	San Francisco, Calif.	2	5	3.2

[a] ATCC, American Type Culture Collection; CIP, Collection of the Institut Pasteur.

ued for an additional 30 min. Sodium perchlorate (5 M) was then added to the lysate in a ratio of 1:5, and the DNA was extracted twice with phenol and twice with chloroform. The DNA was precipitated with isopropanol, washed five times in ice-cold 70% ethanol, and dried before being suspended in endotoxin-free water.

Agarose gel electrophoresis. Purified DNA from *H. ducreyi* was digested with either *Cla*I, *Eco*RI, *Hin*cII, *Hin*dIII, or *Hin*fI according to the protocol of the supplier (U.S. Biochemical, Cleveland, Ohio). Digested DNA was separated by electrophoresis on a 1% agarose gel overnight at 1 V/cm in Tris-borate buffer and transferred onto nylon filters (Micron Separations, Inc., Westboro, Mass.) by the method of Southern (19). A 1-kb DNA ladder (BRL Life Technologies, Inc., Gaithersburg, Md.) was used to provide molecular size markers.

Radioactive labeling of RNA and DNA. 16S and 23S rRNA from *E. coli* (Boehringer Mannheim) was partially hydrolyzed, and the hydrolysis products were 5′ end labeled with [γ-^{32}P]ATP (New England Nuclear Research Products, Wilmington, Del.) (1). The 1-kb DNA ladder (BRL) was nick translated with a nick translation kit (BRL) and [α-^{32}P]dCTP (New England Nuclear Research).

Hybridization. Southern blots of the DNA digests were hybridized with labeled rRNA as described by Altwegg et al. (1). After hybridization, the nylon filters were washed twice in 2× SSC containing 0.1% SDS and twice in 0.25× SSC containing 0.1% SDS at 37°C. Ribosomal DNA (rDNA) banding patterns were visualized after autoradiographs were made with Kodak X-Omat film (Eastman Kodak Co., Rochester, N.Y.).

DNA fragment size determination. The molecular sizes of the restriction fragments were calculated by the method of Southern (19) with the 1-kb ladder bands as reference.

TABLE 2. Characterization of 32 isolates of *H. ducreyi* collected in Orange County, Calif. (18 isolates) and Nairobi, Kenya (14 isolates)

Geographic origin	Yr(s)	No. of isolates	Ribotype		Plasmid size(s) (MDa)
			*Hin*cII	*Hin*dIII	
Orange County	1981 and 1982	14	2	5	3.2
		3	2	5	No plasmid
		1	2	7	3.2
Kenya	1984	5	1	2	7.0
		1	1	3	7.0
		2	2	4	5.7
		1	3	8	7.0
		3	4	6	5.7
		1	4	6	7.0
		1	4	6	4.5, 7.0

RESULTS

Restriction endonuclease digestion. The DNA from *H. ducreyi* ATCC 33922 and C148 was digested with *Eco*RI, *Cla*I, *Hin*cII, *Hin*dIII, or *Hin*fI restriction endonucleases. *Hin*cII or *Hin*dIII digestion resulted in fragments in the range of approximately 1 to 15 kb. *Hin*fI digestion resulted in fragments smaller than 3 kb, whereas *Eco*RI or *Cla*I digestion resulted either in incomplete digestion or in the generation of primarily large fragments. Consequently, all subsequent DNA digestions were performed with either *Hin*cII or *Hin*dIII.

Digestion with *Hin*cII or *Hin*dIII resulted in the production of at least 60 fragment bands as visualized after agarose gel electrophoresis. Three distinct patterns were observed on visual inspection of *Hin*cII digests; most of the variations occurred among fragments corresponding to approximately 3 to 11 kb (Fig. 1A). In contrast, the patterns obtained with *Hin*dIII appeared to be very similar, with the exception of a band corresponding to ca. 4.8 kb and two bands significantly larger than 12 kb (Fig. 1B).

Ribotyping. The ability of *E. coli* rRNA to hybridize with *Hin*cII- or *Hin*dIII-digested DNA was tested. Two distinct ribotype patterns were observed with *Hin*cII-digested DNA among a group of 13 isolates of *H. ducreyi*, including the type strain and strains from North America and Kenya (Fig. 2A and Table 1). The rDNA-containing fragments ranged in size between 0.8 and 11.6 kb, respectively. The two patterns were strikingly similar, with common rDNA-containing fragments corresponding to approximately 0.8, 1.0, 1.3, 1.5, 1.9, 2.1, 2.8, 3.1, 5.6, 5.9, and 6.3 kb. The two ribotype patterns differed in the presence or absence of two rDNA-containing fragments corresponding to approximately 5.0 and 11.6 kb. In contrast, five distinct ribotype patterns were obtained with *Hin*dIII-digested DNA (Fig. 2B and Table 1). The rDNA-containing fragment sizes ranged from 1.1 to 10.6 kb. The five patterns possessed seven common rDNA-containing fragments, which corresponded to sizes of approximately

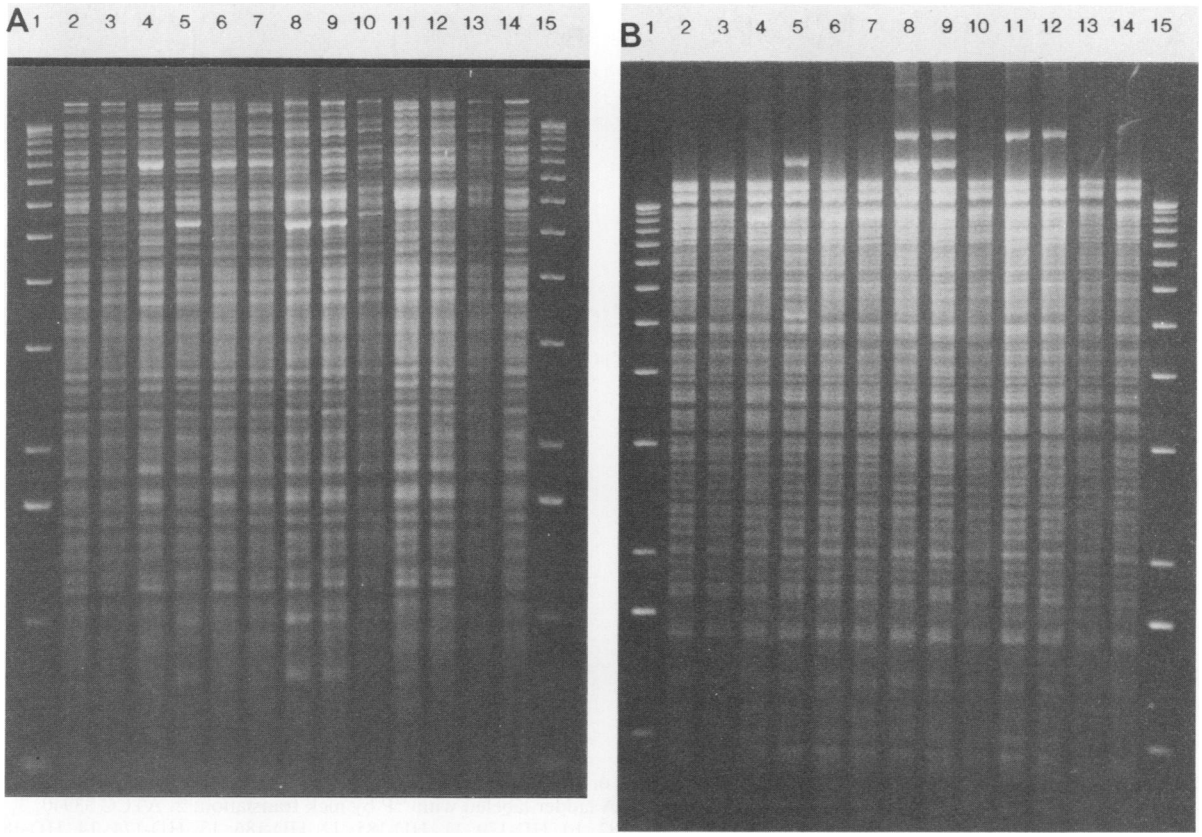

FIG. 1. Agarose gel electrophoresis of *H. ducreyi* DNA digested with *Hinc*II (A) or *Hin*dIII (B) and stained with ethidium bromide. Lanes: 1 and 15, 1-kb DNA ladder; 2, ATCC 33940; 3, ATCC 33922; 4, V1159; 5, C148; 6, HD-187; 7, HD-188; 8, HD-181; 9, HD-182; 10, HD-179; 11, HD-185; 12, HD-186; 13, HD-174; 14, HD-189.

1.1, 1.6, 1.8, 2.1, 3.6, 3.0, and 10.6 kb, respectively. Four rDNA-containing fragments were responsible for the differences among the five patterns; these fragments were approximately 2.6, 4.3, 4.6, and 8.0 kb in size.

The utility of ribotyping as a tool to study the epidemiology of chancroid was further evaluated by comparing the ribotypes and plasmid profiles for 18 isolates from Orange County, Calif., and 14 isolates from Kenya (Table 2). All 18 *H. ducreyi* isolates from the Orange County epidemic belonged to ribotype *Hinc*II-2. Seventeen of these isolates belonged to ribotype *Hin*dIII-5. The rDNA banding pattern of the remaining isolate was identical to that of the 17 isolates with the exception of one band; this new ribotype pattern was designated *Hin*dIII-7. In contrast, the *H. ducreyi* isolates from Kenya, where chancroid is endemic, belonged to ribotypes *Hinc*II-1 and -2 as well as to two new ribotypes designated *Hinc*II-3 and -4. The Kenyan isolates belonged to the *Hin*dIII ribotypes 2, 3, 4, and to 2 new ribotypes designated *Hin*dIII-6 and -8. The banding patterns characteristic of all of the ribotypes are schematically represented in Fig. 3.

Plasmid content. Of the 13 isolates of *H. ducreyi* initially examined, 5 belonged to the ribotype *Hinc*II-1/*Hin*dIII-2 (Table 1). Two of these isolates possessed a 5.7-MDa plasmid, whereas the remaining three possessed a 7.0-MDa plasmid. Similarly, three isolates belonged to the ribotype *Hinc*II-2/*Hin*dIII-4 (Table 1). One of these isolates appeared plasmidless, and the remaining two possessed both 4.5- and 5.7-MDa plasmids.

Five isolates of *H. ducreyi* from Kenya belonged to the ribotype *Hinc*II-4/*Hin*dIII-6 (Table 2). Three of these isolates possessed a 5.7-MDa plasmid, whereas the remaining two possessed either a 7.0-MDa plasmid or both 4.5- and 7.0-MDa plasmids. Only three isolates of *H. ducreyi* from Orange County did not possess a 3.2-MDa plasmid (Table 2).

DISCUSSION

DNA fingerprints derived from rRNA cistrons should provide a novel method of differentiating among strains of *H. ducreyi*. Five restriction enzymes were initially evaluated for their ability to digest DNA from 13 *H. ducreyi* isolates, with the production of fragments with a wide range of sizes. Digestion with *Hin*fI, *Eco*RI, or *Cla*I resulted in fragments that were either too small or too large, as visualized on agarose gels, for use in ribotyping. Complete digests of *H. ducreyi* DNA that were suitable for ribotyping were obtained with *Hinc*II and *Hin*dIII. *Hinc*II-digested DNA yielded four ribotype patterns when Southern blots were probed with *E. coli* 16S and 23S rRNA; eight different ribotype patterns were observed with *Hin*dIII-digested DNA (Tables 1 and 2). The patterns were not influenced by the presence of plasmids or the type of plasmid present in *H. ducreyi* (Tables 1 and 2). In addition, the differences in the restriction fragment patterns observed with *Hinc*II- or *Hin*dIII-digested DNA (Fig. 1) did not correlate with the ribotype patterns (Table 1). When the patterns obtained after *Hin*dIII digestion were compared, it was interesting to note that the pairs of isolates

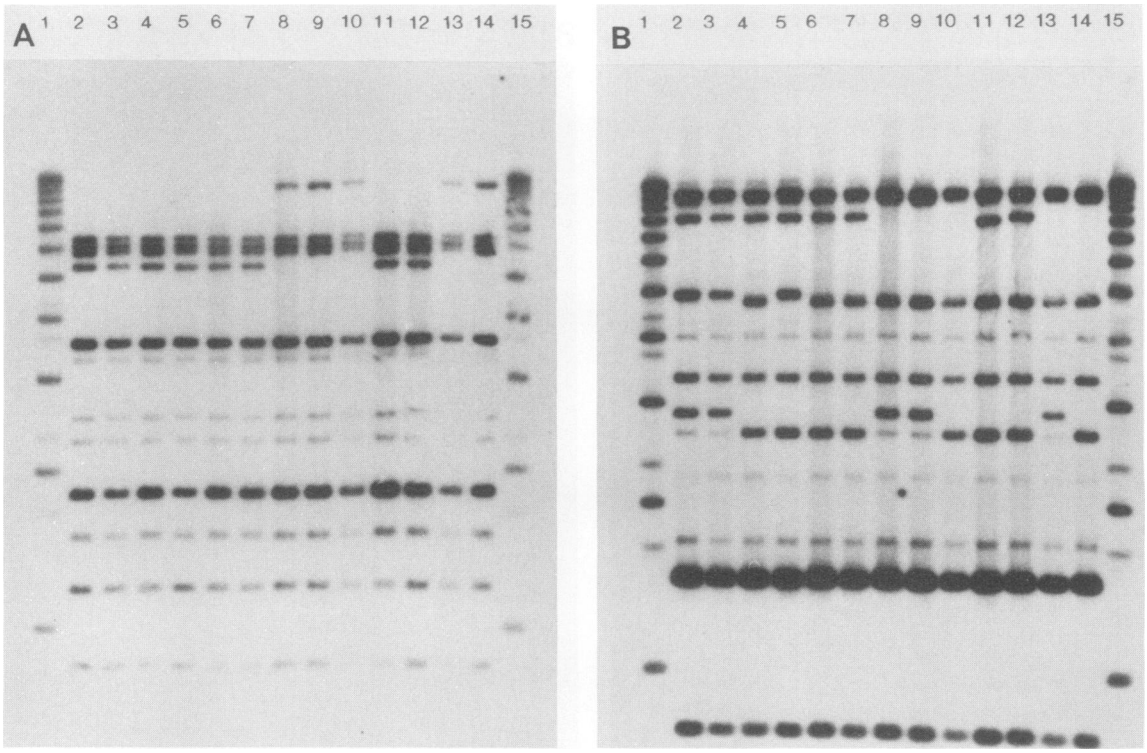

FIG. 2. Southern blots of *H. ducreyi* DNA, digested with *Hin*cII (A) or *Hin*dIII (B), separated by agarose gel electrophoresis, and hybridized with ^{32}P-labeled 16S and 23S *E. coli* rRNA. Lanes: 1 and 15, 1-kb DNA ladder labeled with ^{32}P by nick translation; 2, ATCC 33940; 3, ATCC 33922; 4, V1159; 5, C148; 6, HD-187; 7, HD-188; 8, HD-181; 9, HD-182; 10, HD-179; 11, HD-185; 12, HD-186; 13, HD-174; 14, HD-189.

from Florida, Georgia, and Kenya had identical ribotype patterns (Table 1) and yet were different from one another. Arabic numerals have been assigned to the ribotypes (Fig. 3) obtained with *Hin*cII- or *Hin*dIII-digested DNA.

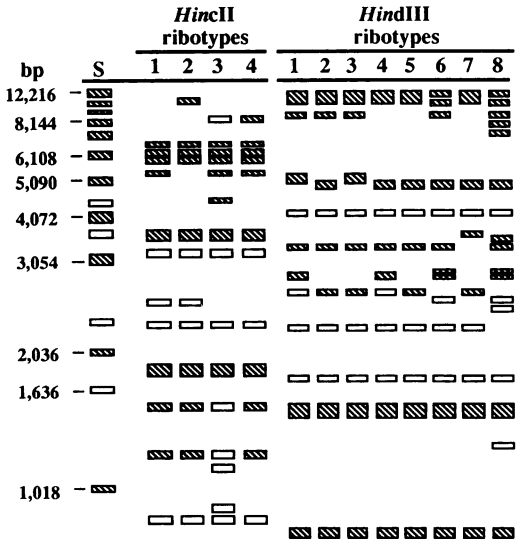

FIG. 3. Ribotype patterns obtained with *Hin*cII- or *Hin*dIII-digested DNA from 44 isolates of *H. ducreyi*. The ribotypes are numbered as indicated above the lanes. S, 1-kb DNA ladder. The shading is a graphical representation of the observed intensity; hatched bands were more intense than blank bands.

The results obtained with the 13 isolates initially examined suggested that ribotyping may be used to differentiate among isolates of *H. ducreyi*. To examine the potential utility of ribotyping in the study of the epidemiology of chancroid, we selected two sets of *H. ducreyi* isolates for ribotype analysis; one set consisted of isolates from Kenya, where chancroid is endemic, and the second set consisted of isolates from a chancroid epidemic that occurred in Orange County, Calif., in 1981 and 1982 (4). It is important to note that the 14 isolates from Kenya were collected within a period of 1 month and consisted of four *Hin*cII ribotypes and five *Hin*dIII ribotypes. Thus, there appears to be a wide diversity of ribotypes in a chancroid-endemic area. In contrast, the isolates from the Orange County outbreak consisted of one *Hin*cII ribotype and two *Hin*dIII ribotypes. Although 17 of 18 isolates belonged to ribotype *Hin*dIII-5, one belonged to ribotype *Hin*dIII-7. *Hin*dIII ribotypes 5 and 7 (Fig. 3) differed by only one fragment, in the range of 3.4 to 3.6 kb; the significance of this difference is presently not understood. The ribotyping results suggest that the Orange County outbreak was due to one or possibly two strains of *H. ducreyi*. However, this remains speculative based on the limited number of isolates examined. Nevertheless, this hypothesis is strengthened by the description of the epidemiology of this epidemic (4). *H. ducreyi* was recovered from the lesions or inguinal buboes of 271 patients. Men accounted for 266 (98%) of the confirmed cases; 95% of the men were hispanic, and at least 53% reported sexual contact with a prostitute. Furthermore, all five culture-positive women were prostitutes. The analysis of the epidemic in Orange County was retrospective and was consequently

limited by the availability of a relatively small proportion of the isolates recovered during the outbreak and by the lack of information regarding the number of times the isolates had been subcultured under nonselective conditions. The latter may be of particular importance when screening for plasmids. For instance, some penicillinase-producing *Neisseria gonorrhoeae* strains have been shown to lose their β-lactamase plasmid under nonselective conditions during growth in continuous culture or upon subculturing in batch culture (9, 16). It was therefore difficult to determine whether the three isolates from Orange County that did not possess the 3.2-MDa β-lactamase plasmid (3) represented different strains.

The use of a second enzyme, *Hinc*II, which resulted in only four ribotypes, provided no additional resolution, because we found no examples among the 44 strains examined in this study of a *Hind*III ribotype that could be distinguished by *Hinc*II ribotyping, although the converse was not true. The *Hinc*II and *Hind*III ribotypes of these strains were mutually exclusive. Ribotypes *Hind*III-1, -2, and -3 correlated with ribotype *Hinc*II-1, whereas ribotypes *Hind*III-4, -5, and -7 correlated with ribotype *Hinc*II-2. Ribotype *Hind*III-6 correlated with ribotype *Hinc*II-4, and ribotype *Hind*III-8 correlated with ribotype *Hinc*II-3. Recently, we identified a ribotype *Hind*III-2 strain from Thailand that belonged to a new *Hinc*II ribotype designated *Hinc*II-5 (data not shown). This suggests that *Hinc*II may provide additional resolution as further strains are examined. Rossau et al. (17) recently reported the majority of the sequence of the 16S rRNA gene and part of the sequence of the 23S rRNA gene of *H. ducreyi* CIP 542. Based on oligonucleotide probes from the sequence of the rRNA genes, it was determined that there was a higher degree of sequence variability among strains of *H. ducreyi* in a region of the 23S rDNA gene. It remains to be determined whether this variability is related to the *Hinc*II or *Hind*III ribotypes we observed in this study.

Various methods have been examined for their ability to differentiate either among strains of *H. ducreyi* or between *H. ducreyi* and other *Haemophilus* spp. Korting et al. (10) used 14 lectins and observed 20 agglutination patterns among 43 *H. ducreyi* isolates from different geographic areas. The stability of the carbohydrate cell surface components that bind these lectins has not been demonstrated; moreover, *H. ducreyi* agglutinates spontaneously when suspended in buffer or medium (12). Thus, the utility of this method for epidemiologic purposes is uncertain. Odumeru et al. (13) used SDS-polyacrylamide gel electrophoresis and observed seven electrophoretic patterns, based on outer membrane proteins, among 105 *H. ducreyi* isolates. Taylor et al. (22) used this technique to characterize strains isolated in Southeast Asia, in which chancroid is endemic. These investigators observed that the differences between some of the types were small and not clear cut; moreover, the distribution of these patterns was limited, suggesting that this method did not discriminate sufficiently to be a useful method for typing *H. ducreyi* isolated in an endemic area. Using an indirect immunofluorescence assay, Slootmans et al. (18) divided 16 strains of *H. ducreyi* into nine types, designated I through IX. The use of indirect immunofluorescence requires the identification of type strains and the production of type-specific antisera. There are few biochemical characteristics that can be used to differentiate among strains of *H. ducreyi*. Van Dyck and Piot (24) used the API-ZYM system to determine the enzyme profiles of 200 strains of *H. ducreyi* isolated in different parts of the world; only three biovars were identified.

The analysis of chromosomal DNA provided a method that may be used to study the relatedness of *H. ducreyi* strains; easily interpretable results were achieved when this was coupled with the detection of restriction fragment length polymorphisms containing rRNA genes. In contrast to the genetic diversity of the remainder of the chromosome (7, 20), a high degree of homology exists in the DNA sequences encoding rRNA. Because rRNA has been more highly conserved during evolution than the rest of the genome (7), the advantage of ribotyping over the use of randomly cloned DNA fragments is that a single probe can be used to study rRNA gene restriction fragments from many different bacterial species. rRNA from *E. coli* is a broad-spectrum probe that has been used in the study of the molecular epidemiology of different bacteria (14, 20). The analysis of rRNA gene restriction patterns has been successfully applied to several bacterial species, including *H. influenzae* biogroup aegyptius (8), *Acholeplasma laidlawii*, *Mycoplasma hominis*, and *Mycoplasma pneumoniae* (25).

The results obtained in this limited study suggest that ribotyping may be useful in the characterization of isolates of *H. ducreyi*. Additional isolates must be tested and other enzymes must be evaluated to determine whether ribotyping provides sufficient discrimination to be useful as a typing system for *H. ducreyi*. We expect that other ribotypes will be identified as additional isolates of *H. ducreyi* from chancroid-endemic areas are examined. Ribotyping does present some disadvantages; it is labor intensive and requires costly enzymes and materials. Nevertheless, ribotyping provides a highly reproducible and reliable reference typing system. Further studies will confirm the utility of ribotyping as a molecular epidemiological tool for studying the distribution of *H. ducreyi* strains. With a caveat concerning plasmid stability, plasmid content (23) may be used with ribotypes of *H. ducreyi* strains to provide additional differentiation among *H. ducreyi* isolates. The use of plasmid type in conjunction with ribotype provided additional differentiation among some isolates belonging to the same *Hinc*II/*Hind*III ribotype (Tables 1 and 2). The development of a stable typing system will permit us to address specific questions concerning the geographical distribution of *H. ducreyi* ribotypes, mode of transmission, treatment failure versus reinfection, virulence, and genetic diversity among *H. ducreyi* isolates.

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