INFECTION AND IMMUNITY, Feb. 1992, p. 590–595
0019-9567/92/020590-06$02.00/0

Vol. 60, No. 2

Antigenic Diversity in *Haemophilus ducreyi* as Shown by Western Blot (Immunoblot) Analysis

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Received 21 June 1991/Accepted 11 November 1991

The antigenic diversity within a panel of 63 *Haemophilus ducreyi* isolates was examined by Western blot (immunoblot) analysis with a pool of 238 well-characterized human antisera. When a serum pool adsorbed on a mixture of *Haemophilus influenzae*, *H. parainfluenzae*, and *H. parahaemolyticus* was used, the immunoprofiles suggested that prominent antigenic proteins involved in the human immune response have apparent molecular masses of 63, 42, 34 to 30, and 28.5 to 28 kDa. Preliminary subcellular localization revealed that these antigens are associated with the cellular membrane. Two subsets of antigens were discriminated by detergent extraction. There was no evidence that the antigen composition is altered by changing the growth conditions. With a serum pool adsorbed on the *Haemophilus* spp. mixture supplemented with *Actinobacillus actinomycetemcomitans*, *Pasteurella ureae*, *Neisseria gonorrhoeae*, and *Escherichia coli*, antigenic determinants more specific for *H. ducreyi* were identified. An immunodominant 28.5- to 28-kDa protein was expressed by all *H. ducreyi* isolates. In the range from 34 to 30 kDa, 56 isolates revealed a dominant protein with variable molecular mass. By using both proteins (28.5 to 28 kDa and 34 to 30 kDa) as immunotypic markers, seven different immunopatterns were identified. Antigenic diversity among isolates from different geographical origins as well as from a single area was observed.

Haemophilus ducreyi is a poorly known and fastidious microorganism causing chancroid, a sexually transmitted disease (STD) that is characterized by genital ulcers and by abcedation of the inguinal lymph nodes (6). Recently, chancroid has received more interest because it is emerging as the major cause of genital ulcer disease in several parts of the developing world, and it is reappearing in poor minority populations in Europe and the United States (10, 14, 16). In addition, several reports suggest that genital ulcer disease, in particular chancroid, is a risk factor for the sexual transmission of the human immunodeficiency virus (9). Little is known about the antigenic composition of *H. ducreyi*, and the nature of the immune response upon infection has been poorly studied. Serum immunoglobulin G (IgG) and IgM antibodies have been detected in patients with a clinical diagnosis of chancroid both by dot immunobinding and by an enzyme immunoassay (8, 13). Western blot analysis of sera from normal and immunized rabbits has shown that rabbits immunized with *H. ducreyi* respond with a humoral immune response in which multiple antigenic polypeptides are detected. The most prominent antigens are reported to have apparent molecular masses of 79, 62, 55, 49, and 26 kDa (7) and of 67, 42, 22.5, and 20 kDa (12). However, the specificity of the antigens towards *H. ducreyi* has not been demonstrated.

In the present study, the antigenic diversity occurring within a panel of *H. ducreyi* isolates was examined by Western blot (immunoblot) analysis. Prominent proteins involved in the human immune response and *H. ducreyi*-specific antigens were identified and localized. The dependency of the immunoprofiles on growth conditions was assessed. On the basis of the immunotypic proteins, classification of the *H. ducreyi* isolates resulted in seven immunotypes. The information may be used for epidemiological purposes, for the development of diagnostic tests, and for the study of the human immune response to infection with this microorganism.

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MATERIALS AND METHODS

Bacterial isolates. *H. ducreyi* isolates were grown on both MHVI agar (Mueller-Hinton agar [BBL] with 5% [vol/vol] fetal calf serum [GIBCO], 5% [vol/vol] chocolatized horse blood [GIBCO], 1% [vol/vol] IsoVitaleX [BBL], and 3 μg of vancomycin [BBL] per ml) and on GCF agar (gonococcal agar [GIBCO] with 1% [wt/vol] bovine hemoglobin [Bethesda Research Laboratories], 5% [vol/vol] fetal calf serum [GIBCO], 1% [vol/vol] IsoVitaleX [BBL], and 3 μg of vancomycin [BBL] per ml). Incubation was for 3 days at 35.5°C in a humidified CO_2 atmosphere (Anaerobic GasPak System [BBL]) for MHVI medium and in a humidified aerobic milieu containing 5% [vol/vol] CO_2 for GCF medium. *Haemophilus influenzae* type b (ITG 3877), *Haemophilus parainfluenzae* (ITG 1094), and *Haemophilus parahaemolyticus* (ITG 402) were grown on GCF agar (10 plates per strain) for 48 h at 35.5°C and 5% [vol/vol] CO_2, as described for *H. ducreyi*. *Neisseria gonorrhoeae* (ITG 4446, ITG 4447, and ITG 4451), *Actinobacillus actinomycetemcomitans* (ITG 1460), and *Pasteurella ureae* (ITG 3842) were grown on GC agar (Difco) supplemented with 1.7% [wt/vol] Bacto-Hemoglobin (Difco) for 48 h at 35.5°C. *Escherichia coli* 0124 was suspended in liquid broth (0.5% [wt/vol] yeastolate [Difco], 1.0% [wt/vol] sodium chloride [Merck], and 1.0% [wt/vol] Bacto-Trypton [Difco]). Incubation was in 50 ml of liquid broth for 18 h at 37°C.

Preparation of antigen. Microorganisms were harvested in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.0, and, like *E. coli*, were sedimented by centrifugation for 15 min at 1,200 × *g*. The pelleted cells were washed twice with 50 ml of buffer and were stored overnight at −20°C. The next day, they were thawed and resuspended in 10 mM HEPES, pH 7.0. One-milliliter fractions of the cell suspensions were sonicated four times (20 s

each) on ice. Sonication was performed with a Branson Sonifier 250 (duty cycle, 90; output, 1; diameter of the probe, 3 mm). The homogenates were centrifuged for 15 min at 1,200 × *g* to remove intact cells. The supernatants resulting from the suspensions of *H. influenzae*, *H. parainfluenzae*, and *H. parahaemolyticus* as well as the supernatants resulting from the suspensions of *E. coli*, *N. gonorrhoeae*, *P. ureae*, and *A. actinomycetemcomitans* were pooled. To these pools, hereafter referred to as the adsorbent and the supplemented adsorbent, respectively, sodium dodecyl sulfate (SDS) was added to a final concentration of 1% (wt/vol). Like the individual SDS-free *H. ducreyi* supernatants, the adsorbents were stored at −20°C in 500-μl aliquots with a protein concentration of 10 mg/ml. The protein content of antigen and adsorbent preparations was determined according to the method described by Bradford (2).

Subcellular fractionation. To assess the localization of the *H. ducreyi*-specific antigens, a total membrane fraction was obtained from the homogenate by ultracentrifugation for 2 h at 200,000 × *g*. Subsequently, this crude membrane fraction was extracted with 2% (vol/vol) Triton X-100 in the presence of $MgCl_2$ (1 mM) for 1 h at room temperature. The insoluble membrane fraction was recovered by ultracentrifugation as described before. The protein content of the individual fractions was then determined (2).

Serum pool. A serum pool was composed by using equal volumes (100 μl) of 238 human serum samples. These sera were from patients with culture-proven chancroid as well as from patients at STD clinics who were seropositive, as demonstrated by an experimental enzyme-linked immunosorbent assay (ELISA) for the detection of IgG anti-*H. ducreyi* antibody (8). The specificity of this assay for the diagnosis of acute chancroid in men and women is estimated at 98%. The selected sera originated from Kigali, Rwanda; The Gambia; Bangkok, Thailand; and Kinshasa, Zaire.

Preadsorption of the serum pool. A 75-μl portion of the serum pool was incubated with 1.5 mg of adsorbent in a final volume of 50 ml of phosphate-buffered saline containing 0.05% (vol/vol) Tween 80. Incubation was for 1 h at room temperature. Large aggregates were sedimented by centrifugation for 5 min at 10,000 × *g*. The supernatant was diluted to 150 ml, and another 1.5 mg of adsorbent was added. Incubation was allowed to proceed for 18 h at 4°C. The following day, residual aggregates were pelleted and the supernatant was immediately used for Western blot analysis. The effectiveness of preadsorption was demonstrated by the absence of immunoreactivity when preadsorbed pools of sera from Belgian STD patients and from African patients without STD were used for Western blot analysis on 20 randomly chosen *H. ducreyi* isolates.

Immunoblotting. The electrophoretic separation of proteins was carried out with a Pharmacia Gel Electrophoresis Apparatus GE-2LS. After reduction, the proteins were alkylated by 1.5% (wt/vol) iodoacetamide (Sigma). For protein separation, the buffer system of Laemmli (5) was used with 0.75-mm-thick 15% (wt/vol) polyacrylamide gels. Migration of the proteins was allowed to proceed at 20°C for 1 h at 100 V and for 2 h at 220 V. Electrophoretic blotting was performed in an LKB Semy-dry blot cell with an initial current of 0.6 mA/cm^2. The electrotransfer was optimized by slightly adapting the buffer conditions described by Dunn (3). Gels were incubated for 30 min at room temperature with transfer buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3, pH 10.4). Subsequently, the proteins were transferred to a 0.22-μm nitrocellulose sheet for 2 h. After blotting, the nitrocellulose was incubated for 2 h with 75 ml of blocking buffer (PBS plus 0.05% [vol/vol] Tween 80 [Fluka]) and overnight with 75 ml of blocking buffer containing 1% [vol/vol] heat-inactivated fetal calf serum. Antigenic determinants were localized by sequential incubation for 2 h at room temperature with the diluted serum pool and with goat anti-human IgG (Fc) alkaline phosphatase (Bethesda Research Laboratories), diluted 1/3,000 in blocking buffer. Finally, the blot was incubated for 15 min at room temperature with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium from Bethesda Research Laboratories. In between the incubations, unbound antibody was removed by washing the nitrocellulose sheet three times (10 min per wash) with blocking buffer.

RESULTS

Western blot analysis of the *H. ducreyi* isolates by using a *Haemophilus* spp. adsorbed serum pool. Western blot analysis of 63 individual *H. ducreyi* lysates was performed with a pool of human antisera after preadsorption of this pool on the *Haemophilus* spp. adsorbent. *H. ducreyi* isolates revealed three separate clusters of antigenic determinants.

Cluster 1, which was bound by 63- and 42-kDa immunodominant proteins, and cluster 3, which was characterized by several fuzzy immunoreactive bands with molecular masses less than 20 kDa, were found to be very similar among these isolates. Cluster 2 showed interisolate variation and was composed of a 28.5- or 28-kDa protein and a set of proteins with molecular sizes ranging from 34 to 30 kDa. This variation allowed the identification of 13 immunoprofiles, of which one representative each is shown in Fig. 1. The specificity of the emerging immunoprofiles was suggested by the absence of immunoreactivity of the serum with adsorbent antigen and marker proteins on a Western blot.

The proteinaceous character of the antigens in clusters 1 and 2 was suggested by their sensitivity to proteinase K and by the lack of reaction with the periodic acid-Schiff reagent as well as with the biotin-labeled lectin from *Phaseolus vulgaris*. The determinants in cluster 3 were proteinase insensitive, bound lectin, and were positive in the periodic acid-Schiff reaction (data not shown).

Because growth of *H. ducreyi* may be highly dependent on the culture conditions such that some isolates prefer MHVI while others prefer GCF, the effect of these growth conditions on the antigen composition was studied by using six isolates growing on both media. Western blot analysis was performed as described previously, and the results are shown in Fig. 2. Only minor quantitative differences were observed.

Western blot analysis of subcellular fractions of *H. ducreyi*. Total homogenates prepared from *H. ducreyi* isolates 3 to 7 and 11 (Fig. 1) were fractionated, and the subcellular fractions were analyzed by Western blot analysis with a serum pool adsorbed with the *Haemophilus* spp. adsorbent. The immunoprofiles showed that all antigens believed to be involved in the human immune response are associated with the membrane of the microorganism. Extraction of this crude membrane fraction with Triton X-100 and 1 mM $MgCl_2$ revealed that two antigen subsets exist. The 63-kDa and 28.5- to 28-kDa antigens were released under these conditions, while the 42-kDa and 34- to 30-kDa antigens remained membrane bound. This distribution of antigens was observed for all six *H. ducreyi* isolates but is shown only for isolate C90/81 (isolate 5 in Fig. 1) (Fig. 3).

Western blot analysis of the *H. ducreyi* isolates by using a serum pool adsorbed on the supplemented adsorbent. In an

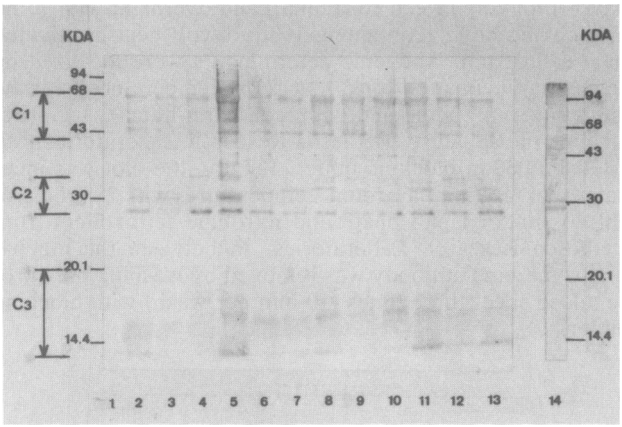

FIG. 1. Immunoprofiles representative of the *H. ducreyi* isolates by using a *Haemophilus* spp. adsorbed serum pool. Protein (15 μg) of the lysed *H. ducreyi* isolates was subjected to Western blot analysis as described in Materials and Methods. The serum pool was adsorbed on the mixture of *Haemophilus* spp. lysates. The emerging immunoprofiles were grouped into 13 immunotypes. For each type, a representative profile is shown. Analysis of the *H. ducreyi* CIP542 antigen required the blot to be incubated for 30 min with alkaline phosphatase substrate for unambiguous immunoprofiles to emerge. Lanes: 1, *Haemophilus* spp. adsorbent; 2, HD47/86; 3, HD699/88; 4, BMC4/89; 5, C90/81; 6, F91/81; 7, PU35/83; 8, PU12/83; 9, 247B1/86; 10, H2/86; 11, HD65/88; 12, C105/81; 13, HD569/87; 14, CIP542/54. Origin and year of isolation of the individual isolates are given in Fig. 5 (isolates indicated by asterisks). Marker proteins are phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). C1 to C3, clusters 1 to 3 (see Results).

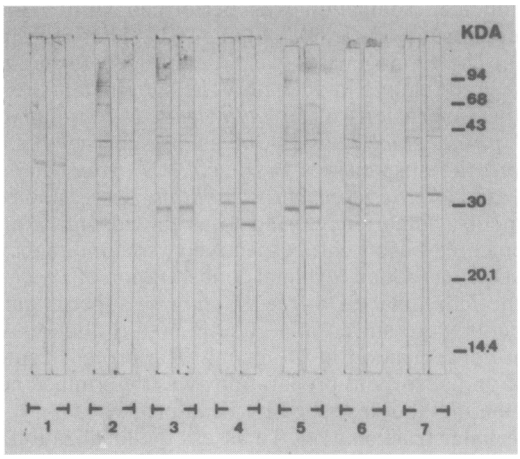

FIG. 2. Assessment of the effect of growth conditions on the immunoprofiles of *H. ducreyi*. Lysed *H. ducreyi* isolates (15 μg) grown on both culture media were subjected to Western blot analysis as described in Materials and Methods. The serum pool was adsorbed on the mixture of *Haemophilus* spp. lysates. The emerging immunoprofiles were compared for both media. Lanes: 1, *Haemophilus* spp. adsorbent; 2, HD47/86; 3, HD699/88; 4, BMC4/89; 5, C90/81; 6, F91/81; 7, PU12/83. (In each set, the left lane is with MHVI medium and the right lane is with GCF medium.) Marker proteins are phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

effort to improve the specificity of the immunoprofiles, Western blot analysis was repeated with a serum pool adsorbed with the supplemented adsorbent. The number of immunoprofiles was reduced to the seven profiles shown in Fig. 4. The immunoreactivity with the 63-kDa immunodominant protein was completely abolished, while the 42-kDa protein was hardly detected. However, the 28.5- and 28-kDa proteins were still recognized. In the supplemented adsorbent, an equivalent protein was detected only weakly. Very pronounced was the effect of extended serum adsorption on the immunodetection of the 34- to 30-kDa proteins. The majority of the immunoprofiles (56 of 63) showed one immunodominant protein in this region. The different molecular sizes observed included 30, 30.5, 31, 32, and 33 kDa.

DISCUSSION

The antigenic determinants of 63 *H. ducreyi* isolates were characterized by Western blot analysis. The specificities of the resulting immunoprofiles were improved by adsorbing the serum pool on a *Haemophilus* sp. adsorbent supplemented with *N. gonorrhoeae*, *A. actinomycetemcomitans*, *E. coli*, and *P. ureae*. Indeed, previous studies have reported cross-reactions of rabbit anti-*H. ducreyi* antisera not only with *Haemophilus* spp. but also with *Pasteurella* sp. and *Actinobacillus* sp. (15). Furthermore, Western blot analysis of *N. gonorrhoeae* and *E. coli* isolates with the human serum pool revealed important immunodeterminants comparable to determinants of *H. ducreyi* as far as molecular weight is concerned (data not shown).

Analysis of a serum pool adsorbed on a *Haemophilus* spp. adsorbent suggested that proteins with molecular masses of 63, 42, 34 to 30, and 28.5 to 28 kDa may play an important role in the human immune response and in the experimental ELISA (8). The abundancy and molecular mass of the larger protein led us to assume the presence of heat-shock proteins in *H. ducreyi*. Further study is in progress to assess this possibility.

Of proteins identified in studies with rabbits, only a 62-kDa (7) and a 42-kDa (12) protein are similar to the antigens identified in this study. This observation stresses the need for careful evaluation of results from studies on animal models before extrapolating to a human model.

A comparative study of the antigen composition of isolates grown on both MHVI and GCF culture media suggested that the immunopatterns were unaffected by changes in growth conditions. This was supported by the absence of any correlation between growth condition and antigenic diversity. A similar observation was reported by Abeck et al. (1). These investigators noticed that lipopolysaccharide patterns, but not protein patterns, were dependent on medium composition, atmospheric conditions, and temperature. We were not able to assess the effect of temperature on the immunoprofiles because none of the isolates grew at temperatures outside the range of 30 to 35°C.

Preliminary localization of the antigens identified in *H. ducreyi* suggested that all important antigens are associated with the membrane fractions but that the 63- and 29-kDa antigens are associated in a way different from that of the other antigens. As far as the larger protein is concerned, molecular weight, localization, and extractability by Triton X-100 suggested the presence of a *Chlamydia trachomatis*-like, membrane-bound, GroEL-class antigen in *H. ducreyi* (17). This issue now is under investigation.

Most of the *H. ducreyi* isolates (57 of 63) expressed a

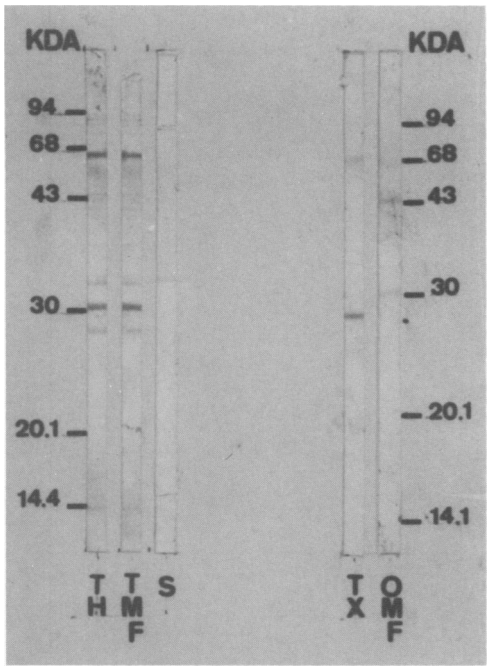

FIG. 3. Preliminary localization of *H. ducreyi* antigens. Individual subcellular fractions (15 μg) were analyzed by Western blotting with a serum pool adsorbed on the *Haemophilus* spp. mixture. The immunoprofiles shown were obtained with isolate C90/81 (Fig. 1). TH, total homogenate; S, soluble fraction; TMF, total membrane fraction; TX, Triton X-100 extract; OMF, outer membrane fraction (Triton X-100-insoluble fraction). Marker proteins are phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

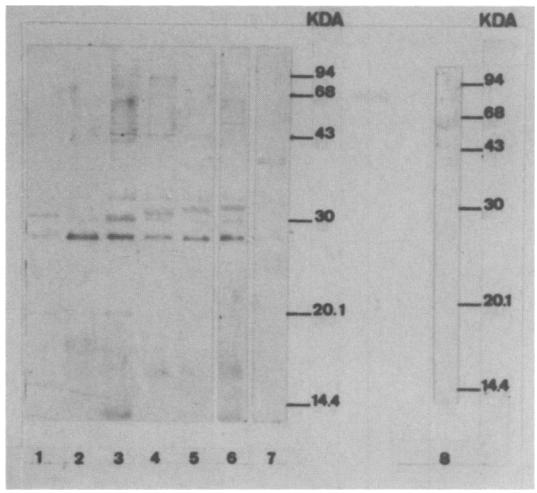

FIG. 4. Immunoprofiles representative of the *H. ducreyi* isolates by using the serum pool adsorbed on the supplemented adsorbent. Protein (15 μg) of the lysed *H. ducreyi* isolates was subjected to Western blot analysis as described in Materials and Methods. The serum pool was adsorbed on the mixture of *Haemophilus* spp. lysates supplemented with a mixture of *N. gonorrhoeae*, *A. actinomycetemcomitans*, *P. ureae*, and *E. coli* lysates. The emerging immunoprofiles were grouped into seven immunotypes. For each type, a representative profile is shown. For analysis of the *H. ducreyi* CIP542 antigen, the blot was incubated for 30 min with alkaline phosphatase substrate. Lanes: 1, HD699/88; 2, BMC4/89; 3, C90/81; 4, F91/81; 5, PU35/83; 6, HD65/88; 7, supplemented adsorbent; 8, CIP542/54. Origin and year of isolation of the individual isolates are given in Fig. 5 (isolates indicated by open circles). Marker proteins are phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

28-kDa protein, while a few (5 of 63) produced a 28.5-kDa protein instead. Since the immunoreactivity of the serum pool with these determinants was not negatively affected by either adsorbent and since in neither adsorbent an equivalent protein showed similar immunodominance, these proteins were considered to be species specific. Recently, Karim and coworkers (4) have reported the production of a monoclonal antibody directed against a 29-kDa protein and reactive with all strains of *H. ducreyi*. Although the relation between the two reported proteins remains to be demonstrated, our observation supports their usefulness as species-specific markers.

Great interisolate variation was observed among the antigens of the 34- to 30-kDa cluster. By using a serum pool adsorbed on the supplemented adsorbent, the complexity of this cluster was reduced to a single immunodominant protein, with variable molecular mass, present in 56 isolates. Whether the variation is due to expression of different antigens or to size variation of the same determinant is not yet clear. Whatever the reason, the appearance of this dominant protein was highly reproducible; consequently, the protein may be used as an immunotypic marker (Fig. 5).

In this study, 63 *H. ducreyi* isolates were classified according to the pattern of the 28.5- to 28-kDa and 34- to 30-kDa proteins. The classification is correlated with the origin and year of collection. Though further analysis is required, the classification suggests that there is as much antigenic variation among isolates from one geographical origin as there is among isolates from different areas. Recently, Sarafian and coworkers (11) reported that differences in genomic fingerprints could be used for typing *H. ducreyi* isolates. How this typing correlates with the immunotyping proposed in this report needs to be investigated.

A low immunoreactivity between reference strain CIP542 (immunotype G) and adsorbed serum was observed. This observation may indicate that, since its isolation in 1954, the antigenic characteristics of this isolate have changed significantly because of cultivation in the laboratory, that this isolate is atypical, or that *H. ducreyi* as a species has antigenically shifted over the years.

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In conclusion, preadsorption of a pool of human sera allowed the identification by Western blot analysis of membrane-bound proteins that may play a role in the humoral immune response after infection by *H. ducreyi* and of species-specific as well as immunotypic markers. Serological classification of 63 *H. ducreyi* isolates yielded seven immunotypes. This classification may be useful for studies on the diagnosis, epidemiology, and pathogenesis of chancroid.

	Immunotypes						
	A	B	C	D	E	F	G
32	—		—				
30		—			—	—	
28	—	—	—	—	—	—	
kDa							
Code of isolates	Kigali, Rwanda HD27/86 *HD47/86 HD197/86 HD262/87 *HD569/87 HD48/88 HD47/88 Bangkok, Thailand PU1/82 PU46/82 BRH109/82 °*PU35/83 *247B1/86 Kinshasa, Zaire Mst701324-010/88 Mst711324-000/88 Mst701047-111/88 Mst701047-121/88 Mst700714-140/88 Mexico HD473/86 Nairobi, Kenya C148/81 HD1353B/82 Goteborg, Sweden CCUG10045	Nairobi, Kenya NC4116/81 G/82 Amsterdam, Netherlands HD6/78 HD8/78 Kigali, Rwanda HD178/86 HD19/88 HD20/88 HD86/88 Bangkok, Thailand 3113/81 PU45/82 PU75/83 *H2/86 Johannesburg, South Africa °*F91/81 M323/81 Carletonville, South Africa C40/81 C33/81 *C105/81 Goteborg, Sweden CCUG14233 Paris, France CIP76	Bangkok, Thailand *PU12/83 BMC1/89 BMC6/89 BMC9/89 BMC12/89 BMC16/89 BR90/89 BR111/89 Kinshasa, Zaire *°HD65/88	Bangkok, Thailand *°BMC4/89 BMC20/89 BMC24/89 BMC29/89 BMC34/89 BR97/89	Kigali, Rwanda HD93/86 HD10/88 Kinshasa, Zaire *°HD699/88 Johannesburg, South Africa M348/81 Bangkok, Thailand BMC35/89	Carletonville, South Africa !*°C90/81 Johannesburg, South Africa GU1/86	Hanoi, Vietnam *°CIP542/54
Total no.	21	19	9	6	5	2	1

ACKNOWLEDGMENTS

This study was supported by a grant from the Swedish Agency for Research Cooperation with Developing Countries (SAREC).

We thank S. De Breucker, D. Hendriksen, and K. Janssens for technical assistance. Biological material was kindly provided by J. Bogaerts, E. Echeverria, D. Dance, M. Laga, W. Albritton, T. Lagergard, A. Sturm, and R. Ballard.

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