Typing of Nonencapsulated *Haemophilus* Strains by Repetitive-Element Sequence-Based PCR Using Intergenic Dyad Sequences

Guillaume Bruant, Stephane Watt, Roland Quentin, and Agnes Rosenau*

Département de Microbiologie Médicale et Moléculaire, Unité de Bactériologie,
Centre Hospitalo-Universitaire Bretonneau, 37044 Tours Cedex, France

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Intergenic dyad sequences (IDS) are short repeated elements that have been described for several *Haemophilus* genomes and for only two other bacterial genera. We developed a repetitive-element sequence-based PCR using an IDS-specific primer as a typing method (IDS-PCR) for nonencapsulated *Haemophilus* strains and compared this technique with pulsed-field gel electrophoresis (PFGE) of DNA restricted with *Sma*I. IDS-PCR was rapid, easy to perform, and reproducible, with a high discriminatory capacity for nontypeable *Haemophilus influenzae* (NTHI) strains. The 69 NTHI strains tested generated 65 different banding patterns. Epidemiologically related strains gave similar or identical fingerprints, and all of the unrelated strains except two showed different patterns. These results were in agreement with those obtained by PFGE. For 20 genital strains usually identified as being biotype IV NTHI and belonging to a cryptic genospecies of *Haemophilus* with remarkable genetic homogeneity, four bands were significantly present and six bands were significantly absent from the fingerprints. The 20 strains were gathered in 11 closely related profiles, whereas PFGE provided no band when DNA was treated with *Sma*I. IDS-PCR improved the differentiation previously obtained within this species by ribotyping and multilocus enzyme electrophoresis. Our findings suggest that IDS-PCR is a rapid, reliable, and discriminatory method for typing NTHI strains and is currently the most efficient method for distinguishing strains within the cryptic genospecies of *Haemophilus*.

Nontypeable *Haemophilus influenzae* (NTHI) strains are human commensal bacteria that are responsible for a number of respiratory tract infections such as otitis media, bronchitis, and sinusitis. NTHI strains are also responsible for genital, mother-infant, and neonatal infections. A group of genital strains usually identified as biotype IV NTHI constitutes a cryptic genital genospecies that shows remarkable genetic homogeneity and forms a monophyletic unit with *H. influenzae* and *Haemophilus haemolyticus* (31, 32).

NTHI strains are extremely diverse, and many typing methods have been proposed for epidemiological studies and pathogenesis investigations. Biotyping and more-discriminatory phenotypic typing methods such as outer membrane protein analysis (2, 25, 26, 33) and lipooligosaccharide analysis (6, 13, 26) have gradually been replaced by genotypic techniques. Multilocus enzyme electrophoresis (MLEE) (27, 30, 31) is a discriminatory technique but is also very laborious. DNA restriction fragment length polymorphism (RFLP) analysis (4, 20, 42) has a high discriminatory capacity but gives overly complex banding patterns. Analysis of rRNA gene restriction patterns (ribotyping) (4, 16, 32, 46) is a discriminatory but time-consuming method. Analysis of pulsed-field gel electrophoresis (PFGE) patterns in which genomic DNA is digested with a rare cutting restriction enzyme (1, 36) is currently considered to be the “gold standard” for *H. influenzae* typing but

is also a laborious and time-consuming method. In comparison to these labor-intensive methods, the analysis of randomly amplified polymorphic DNA (RAPD) profiles (17, 28) is rapid and can provide discriminatory results, particularly when several primer results are combined, but this method lacks reproducibility.

Several short (<200-bp) interspersed noncoding repetitive DNA sequences are widely distributed throughout prokaryotic genomes (21). Among these repeated elements, repetitive extragenic palindromic (REP) sequences and enterobacterial repetitive intergenic consensus (ERIC) sequences were initially identified in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium but are also present in other enterobacterial strains and in numerous eubacterial species (21, 45). Enterobacterial strains have been typed by PCR using REP- and ERIC-specific primers (45). This typing method, called rep-PCR (repetitive-element sequence-based PCR), was rapidly developed and has been successfully applied to other groups of bacteria such as *Staphylococcus aureus* (41), *Streptococcus pneumoniae* (44), *Mycobacterium tuberculosis* (37), and *Listeria monocytogenes* (14).

The typing of NTHI strains using rep-PCR with REP- or ERIC-specific primers has given results consistent with those obtained by classical genotypic methods. rep-PCR assays are quick and inexpensive, are less laborious than ribotyping and PFGE, and generate results that are easier to interpret than those of RFLP and more reproducible than those of RAPD (10, 15, 29, 42). However, Pettigrew et al. recently reported that ERIC-PCR was less discriminatory than ribotyping and PFGE, and they recommended that ERIC-PCR be combined with other typing methods (29). These authors also pointed out

* Corresponding author. Mailing address: Département de Microbiologie Médicale et Moléculaire, EA3250, Unité de Bactériologie, Centre Hospitalo-Universitaire Bretonneau, 37044 Tours Cedex, France. Phone: (33) 2 47 47 80 59. Fax: (33) 2 47 47 38 12. E-mail: rosenau@med.univ-tours.fr.

the lack of true REP and ERIC sequences in the *H. influenzae* genome. The absence of such sequences was shown in the fully sequenced *H. influenzae* strain Rd (8, 29) and was confirmed by a full BLAST search of the sequenced regions of various *Haemophilus* strains in the EMBL and GenBank databases. Thus, low-stringency PCR conditions (up to 5 mM MgCl₂ and annealing temperatures as low as 25°C) or degenerate primers were used in REP- and ERIC-PCR for *H. influenzae* typing. These conditions allowed nonspecific binding and decreased the reproducibility of the method.

rep-PCR using primers derived from genuine *Haemophilus* repeated sequences has not yet been performed for *Haemophilus* strains. Such primers would allow high-stringency PCR conditions, thus avoiding the drawbacks of REP- and ERIC-PCR. Mrázek and Karlin (24) have described a short noncoding repeated sequence that is widely distributed throughout the *H. influenzae* Rd genome and is often found in pairs as inverted repeats (18, 34). This 23-bp sequence, commonly called intergenic dyad sequence (IDS), has been found in several *Haemophilus* strains (3, 9, 22, 35, 43). A full and systematic BLAST search for IDSs in the combined EMBL and GenBank databases produced matches with sequences from only two other bacterial genera, *Neisseria* and *Pseudomonas*.

In the present study, PCR with an IDS-specific primer was used as a typing method for 90 *Haemophilus* strains. We evaluated the reproducibility of this technique, its discriminatory capacity, and its ability to cluster epidemiologically related strains. These findings were compared with those obtained by PFGE of DNA restricted with *Sma*I.

MATERIALS AND METHODS

Bacterial strains. Ninety *Haemophilus* strains were included in this study (Table 1). All clinical isolates were capsule serotyped as previously described by Quentin et al. (31). The absence of capsular genes was confirmed by Southern blotting with capsular-type-specific probes prepared with the primers described by Falla et al. (7). All strains were biotyped with the API 20E system (bioMérieux sa, Marcy l'Etoile, France) as described by Holmes et al. (11). Isolates were tested for urease activity, indole production, and ornithine decarboxylase activity according to Kilian's classification (19). Fifty-four strains were isolated from patients with neonatal, mother-infant, and genital infections (genital strains). Twenty of these strains which were nonencapsulated and phenotypically identified as being biotype IV *H. influenzae* strains had previously been assigned to a cryptic genital genospecies of *Haemophilus* (32). Thirty-two strains were isolated from adults (14 strains) and children (18 strains) aged 1 month to 6 years, mostly with respiratory tract infections (28 strains). All strains were epidemiologically unrelated except for nine isolated from four distinct mother-infant infections. Four reference strains (*H. influenzae* Rd, *H. haemolyticus* CIP 103290, and NTHI strains CIP 102514 and CIP 5424) were also included in this study. All strains were stored at -80°C in Schaedler-vitamin K₃ broth (bioMérieux sa) with 10% glycerol.

Genomic DNA extraction. The bacteria were grown for 24 h on chocolate agar plates (bioMérieux sa) at 37°C under 8% CO₂-92% air. Bacterial cells were scraped from the plates and suspended in 1 ml of TEK buffer (40 mM Tris [pH 8]-2 mM EDTA). After addition of 150 µl of 1% lysozyme (Sigma, St. Louis, Mo.), the mixture was incubated for 30 min at 37°C with gentle agitation. Twenty-five microliters of 1% pronase (Calbiochem-Novabiochem Corporation, La Jolla, Calif.) and 150 µl of 10% sodium dodecyl sulfate (Bio-Rad Laboratories, Hercules, Calif.) were then added, and the mixture was incubated for 60 min at 60°C before undergoing two phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma) extractions. After centrifugation for 15 min at 18,800 × g, the DNA was precipitated in the presence of 30 mM NaCl in absolute ethanol for 15 min at -80°C. After centrifugation, the pellet was suspended in 100 µl of TE buffer (10 mM Tris [pH 8]-1 mM EDTA). DNA was quantified by photospectrometry.

PCR assay. The IDS primer (5' GTAGGGTGGGCGTAAGCCC 3') (Eurogentec, Seraing, Belgium) was designed from the 23-bp repeated sequence of *H. influenzae* previously described by Read and Farley (34). The PCR mixture (20

µl) contained genomic DNA (20 ng), the IDS primer (4 µM), deoxynucleoside triphosphates (100 µM each) (Roche Diagnostics, Mannheim, Germany), *Pfu* Turbo DNA polymerase (1.25 U) (Stratagene, La Jolla, Calif.), 2 mM MgCl₂, 20 mM Tris-HCl (pH 8.8), 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, and 10 mM KCl (Stratagene). The PCR consisted of an initial 2.5-min hold at 94°C; 30 cycles, each consisting of 1 min of denaturation at 94°C, 1 min of annealing at 50°C, and 2 min of elongation at 72°C; and a final 10-min elongation step (GeneAmp PCR System 9600; Perkin-Elmer, Norwalk, Conn.). The PCR products were subjected to electrophoresis on 1% agarose gels, and the resulting patterns were visualized by ethidium bromide staining and photographed.

Reproducibility. To check for reproducibility, IDS-PCR was performed on five separate occasions on 10 *Haemophilus* strains (6 genital strains, 3 respiratory tract strains, and strain Rd). Both the GeneAmp PCR System 9600 and the GeneAmp PCR System 2400 (Perkin-Elmer) were used.

PFGE. PFGE was performed as described by Moor et al. (23). Briefly, bacteria grown on chocolate agar plates (bioMérieux) were suspended in TE buffer, mixed with an equal volume of low-melting-temperature agarose (FMC Bio-Products, Rockland, Maine), and placed in a slot former (Bio-Rad Laboratories). The resulting inserts were placed in 2 ml of lysis solution (0.5 M EDTA-1% Sarkosyl) and after the addition of 10 µl of proteinase K (20 mg/ml) were incubated for 48 h at 37°C. After washes, the DNA was digested overnight with *Sma*I (Roche Diagnostics). Restriction fragments were subjected to electrophoresis in a 1% agarose gel (FMC BioProducts) in TBE (4.5 mM Tris-HCl, 4.5 mM borate, 0.125 mM EDTA [pH 7.7]) with a contour-clamped homogeneous electric field (CHEF DRIII; Bio-Rad Laboratories). Pulse times were ramped from 6 to 8 s for 7 h, followed by a second ramp of 1 to 38 s for 17 h at 6 V/cm. Gels were stained with ethidium bromide, and PFGE patterns were detected by UV transillumination.

Analysis of patterns. Photographs of each gel obtained by IDS-PCR and by PFGE of DNA restricted with *Sma*I were digitized with a video camera connected to a microcomputer (Bio-Profil; Vilber-Lourmat, Marne la Vallée, France). Numerical analysis was carried out with the Taxotron package (Taxolab; Institut Pasteur, Paris, France), which includes the RestrictoScan and RestrictoTyper programs. All of the bands on each gel were taken into account, even the faint ones. The band migration distances for each lane were determined in pixel units with RestrictoScan. The molecular size of each fragment was calculated from the migration distances by using cubic spline algorithms with RestrictoTyper. A 1-kb DNA ladder (Invitrogen Life Technologies, Cergy Pontoise, France) and a Lambda ladder (Bio-Rad Laboratories) were used as molecular weight standards for size determination.

Statistical analysis and discrimination index. To evaluate differences in the presence or absence of DNA fragments in the banding patterns obtained by IDS-PCR with the different groups of strains, the chi-square test or the two-tailed Fisher's exact test was performed using EpiInfo software, version 6.04. *P* values of <0.05 were considered to be statistically significant.

The single numerical discrimination index (*D*) was calculated by the method of Hunter and Gaston to evaluate the discriminatory power of the IDS-PCR and PFGE typing methods (12). The probability of two unrelated strains being placed in different typing groups was calculated by the equation

$$D = 1 - [1/(N(N-1))] \sum_{j=1}^S nj(nj-1)$$

where *N* is the total number of strains in the sample population, *S* is the total number of types described, and *n_j* is the number of strains belonging to the *j*th type.

RESULTS

General characteristics and diversity of the IDS-PCR patterns of the *Haemophilus* strains. PCR with IDS primers produced banding patterns with 2 to 10 fragments ranging from 0.3 to 3.8 kb. The distinct patterns obtained with 18 *Haemophilus* strains are shown in Fig. 1. Among the 69 *H. influenzae* strains studied, 64 non-epidemiologically-related strains gave 63 different patterns. *D* was greater than 0.99. Close or identical patterns were obtained for epidemiologically related strains isolated from patients with neonatal and mother-infant infections (Fig. 2). Two pairs of strains (G6-G7 and G29-G30), each comprising one strain isolated from the mother and

TABLE 1. Characteristics of the *Haemophilus* isolates analyzed in this study

Strain (other denomination)	Species	Biotype	Specimen	Country
Genital strains				
C1 (189)	Cryptic genital <i>Haemophilus</i>		Amniotic fluid	United States
C2 (421)	Cryptic genital <i>Haemophilus</i>		Blood of neonate	United States
C3 (422)	Cryptic genital <i>Haemophilus</i>		Blood of neonate	United States
C4 (427)	Cryptic genital <i>Haemophilus</i>		Amniotic fluid	United States
C5 (799)	Cryptic genital <i>Haemophilus</i>		Blood of neonate	United States
C6 (847)	Cryptic genital <i>Haemophilus</i>		Scrotal abscess	United States
C7 (911)	Cryptic genital <i>Haemophilus</i>		Cerebrospinal fluid of neonate	United States
C8 (1595)	Cryptic genital <i>Haemophilus</i>		Blood of neonate	United States
C9 (1610)	Cryptic genital <i>Haemophilus</i>		Blood of neonate	United States
C10 (3N)	Cryptic genital <i>Haemophilus</i>		Gastric fluid of neonate	France
C11 (10N)	Cryptic genital <i>Haemophilus</i>		Gastric fluid of neonate	France
C12 (12N)	Cryptic genital <i>Haemophilus</i>		Gastric fluid of neonate	France
C13 (15N)	Cryptic genital <i>Haemophilus</i>		Gastric fluid of neonate	France
C14 (16N)	Cryptic genital <i>Haemophilus</i>		Gastric fluid of neonate	France
C15 (10U)	Cryptic genital <i>Haemophilus</i>		Urethra (male)	France
C16 (11PS)	Cryptic genital <i>Haemophilus</i>		Urethra (male)	France
C17 (26E)	Cryptic genital <i>Haemophilus</i>		Uterus	France
C18 (PIZ)	Cryptic genital <i>Haemophilus</i>		Endocervix	France
C19 (2406)	Cryptic genital <i>Haemophilus</i>		Vagina	France
C20 (TRA)	Cryptic genital <i>Haemophilus</i>		Endocervix	France
G1 (NAJ)	<i>H. influenzae</i>	III	Intrauterine device	France
G2 (ROY)	<i>H. influenzae</i>	III	Vagina	France
G3 (BOU)	<i>H. influenzae</i>	III	Endocervix	France
G6 (GAR)	<i>H. influenzae</i>	II	Gastric fluid of neonate	France
G7 (GAR)	<i>H. influenzae</i>	II	Vagina	France
G9 (LOP)	<i>H. influenzae</i>	II	Bartholin's gland abscess	France
G11 (ELA)	<i>H. influenzae</i>	I	Intrauterine device	France
G12 (VIG)	<i>H. influenzae</i>	II	Intrauterine device	France
G13 (BER)	<i>H. influenzae</i>	II	Endocervix	France
G14 (ROB)	<i>H. influenzae</i>	II	Blood of neonate	France
G15 (SAU)	<i>H. influenzae</i>	V	Endocervix	France
G17 (PER)	<i>H. influenzae</i>	III	Intrauterine device	France
G18 (PEA)	<i>H. influenzae</i>	V	Endocervix	France
G19 (CAY)	<i>H. influenzae</i>	II	Vagina	France
G20 (MBA)	<i>H. influenzae</i>	II	Gastric fluid of neonate	France
G21 (GOU)	<i>H. influenzae</i>	I	Vagina	France
G22 (KUP)	<i>H. influenzae</i>	II	Gastric fluid of neonate	France
G23 (VER)	<i>H. influenzae</i>	II	Amniotic fluid	France
G24 (BAU)	<i>H. influenzae</i>	V	Endocervix	France
G25 (BAU)	<i>H. influenzae</i>	V	Placenta	France
G26 (BAU)	<i>H. influenzae</i>	V	Gastric fluid of neonate	France
G27 (SAY)	<i>H. influenzae</i>	III	Bartholin's gland abscess	France
G28 (DAC)	<i>H. influenzae</i>	II	Endocervix	France
G29 (LEL)	<i>H. influenzae</i>	II	Blood of neonate	France
G30 (LEL)	<i>H. influenzae</i>	II	Placenta	France
G31 (PEG)	<i>H. influenzae</i>	III	Endocervix	France
G33 (BLI)	<i>H. influenzae</i>	III	Gastric fluid of neonate	France
G34 (IRL)	<i>H. influenzae</i>	II	Bartholin's gland abscess	France
G35 (HIN)	<i>H. influenzae</i>	I	Gastric fluid of neonate	France
G36 (PLA)	<i>H. influenzae</i>	I	Bartholin's gland abscess	France
G37 (HEN)	<i>H. influenzae</i>	III	Endocervix	France
G38 (VER)	<i>H. influenzae</i>	II	Anus of neonate	France
G39 (BEC)	<i>H. influenzae</i>	II	Endocervix	France
G40 (MER)	<i>H. influenzae</i>	II	Intrauterine device	France
Nongenital strains				
Isolates from children				
CH1 (PRO)	<i>H. influenzae</i>	II	Conjunctiva	France
CH3 (AUD)	<i>H. influenzae</i>	I	External ear	France
CH4 (FER)	<i>H. influenzae</i>	III	Trachea	France
CH5 (FOU)	<i>H. influenzae</i>	III	Middle ear	France
CH6 (GOB)	<i>H. influenzae</i>	II	Nasal swab	France
CH7 (RIB)	<i>H. influenzae</i>	I	External ear	France
CH8 (NGU)	<i>H. influenzae</i>	II	Mouth	France
CH9 (BOU)	<i>H. influenzae</i>	III	Nasal swab	France
CH10 (LOP)	<i>H. influenzae</i>	IV	External ear	France
CH11 (FAG)	<i>H. influenzae</i>	II	Urine	France

Continued on following page

TABLE 1—Continued

Strain (other denomination)	Species	Biotype	Specimen	Country
CH12 (DEW)	<i>H. influenzae</i>	I	External ear	France
CH13 (MAN)	<i>H. influenzae</i>	III	Conjunctiva	France
CH14 (PIL)	<i>H. influenzae</i>	II	Middle ear	France
CH15 (MOU)	<i>H. influenzae</i>	II	Trachea	France
CH16 (ROB)	<i>H. influenzae</i>	III	Middle ear	France
CH17 (FOR)	<i>H. influenzae</i>	IV	Middle ear	France
CH18 (COR)	<i>H. influenzae</i>	II	Throat abscess	France
CH19 (COE)	<i>H. influenzae</i>	I	Cerebrospinal fluid	France
Isolates from adults				
A1 (REB)	<i>H. influenzae</i>	II	Sinus	France
A2 (HUE)	<i>H. influenzae</i>	III	Bronchial aspirate	France
A3 (MER)	<i>H. influenzae</i>	II	Bronchial aspirate	France
A4 (MEZ)	<i>H. influenzae</i>	II	Bronchoalveolar fluid	France
A5 (DUB)	<i>H. influenzae</i>	II	Protected distal brush	France
A6 (FER)	<i>H. influenzae</i>	II	Bronchial aspirate	France
A7 (COL)	<i>H. influenzae</i>	III	Protected distal brush	France
A8 (GUI)	<i>H. influenzae</i>	III	Bronchial aspirate	France
A10 (VOL)	<i>H. influenzae</i>	III	Pelvic abdominal abscess	France
A13 (BLA)	<i>H. influenzae</i>	II	Blood culture	France
A16 (GUI)	<i>H. influenzae</i>	I	Protected distal brush	France
A17 (LEB)	<i>H. influenzae</i>	II	Bronchial aspirate	France
A20 (MIC)	<i>H. influenzae</i>	II	Sinus	France
A21 (LAR)	<i>H. influenzae</i>	II	Bronchial aspirate	France
Reference strains				
CIP 5424	<i>H. influenzae</i>	IV	Unknown	Unknown
CIP 102514	<i>H. influenzae</i>	II	Unknown	United States
CIP 103290	<i>H. haemolyticus</i>		Sputum	United Kingdom
CIP 104746 (Rd)	<i>H. influenzae</i>	II	Unknown	Unknown

one strain isolated from her child, gave identical patterns. One pair (G23–G38) and one group of three strains (G24–G25–G26), isolated from a mother and her child, gave closely related banding patterns. Only two non-epidemiologically-related strains (G22 and G23) gave identical patterns. The 20 non-epidemiologically-related genital strains belonging to the

cryptic genospecies of *Haemophilus* showed 11 closely related patterns ($D = 0.91$) (Fig. 3). Thirteen of these 20 strains were grouped into four banding patterns including 5, 3, 3, and 2 strains, respectively.

Eleven DNA fragments appeared to be significantly associated with one or several groups of strains as determined by the

FIG. 1. Banding patterns obtained by IDS-PCR for 18 *Haemophilus* strains. Lane 1, cryptic genital strain C2; lanes 2 to 8, genital strains G1 and G35 to G40; lanes 9 to 18, nongenital strains A3 to A7 and CH3 to CH7; lane B, reagent blank; lanes M, size markers.

FIG. 2. Banding patterns obtained by IDS-PCR. Lanes 1 and 2, identical profiles of strains G6 and G7; lanes 3 and 4, identical profiles of strains G22 and G23; lanes 5 to 7, closely related profiles of strains G24, G25, and G26; lanes 8 and 9, identical profiles of strains G29 and G30; lanes B, reagent blank; lanes M, size markers.

chi-square test or the two-tailed Fisher's exact test ($P < 0.01$) (Table 2). An intensely staining 1.9-kb DNA fragment and a 0.7-kb fragment were present in all of the 20 cryptic genital strains (Fig. 3) and were found in only 18 and 21 of the 70 other *Haemophilus* (69 *H. influenzae* and one *H. haemolyticus*) strains, respectively ($P \leq 0.0000001$). Two other fragments of approximately 0.9 and 0.5 kb were present in 15 and 13 of the 20 cryptic genital strains, respectively, and were found in only 27 ($P < 0.01$) and 7 ($P < 0.000001$) of the 70 other *Haemophilus* strains, respectively. Five fragments of 1.6, 0.6, 3.3, 1.0, and 2.6 kb were absent from all of the 20 cryptic genital strains and were found in 44 ($P < 0.000001$), 34 ($P < 0.001$), 27 ($P < 0.01$), 27 ($P < 0.01$), and 21 ($P < 0.01$) of the 70 other *Haemophilus* strains, respectively. Two other fragments of approximately 2.2 and 1.4 kb were significantly associated with the noncryptic genital strains. These bands were present in 19 and 21 of the 34 noncryptic genital strains, respectively, and were found in only 9 ($P < 0.001$) and 14 ($P < 0.01$) of the 56 other strains,

FIG. 3. Banding patterns obtained by IDS-PCR with the cryptic genital strains C2, C3, C7, C8, and C10 (lane 1), C4, C6, and C14 (lane 2), C19 (lane 3), C5 (lane 4), C9, C11, and C12 (lane 5), C13 and C20 (lane 6), C17 (lane 7), C1 (lane 8), C15 (lane 9), C16 (lane 10), and C18 (lane 11). Lane B, reagent blank; lanes M, size markers. The sizes of the two fragments common to the 20 cryptic genital strains are indicated on the right.

respectively. The 2.2-kb fragment was absent from all of the 20 cryptic genital strains.

Reproducibility of the IDS-PCR method. To assess whether this technique was reproducible, IDS-PCR was performed on five separate occasions with 10 *Haemophilus* strains. Three genital strains belonging to the cryptic genospecies of *Haemophilus* that showed closely related patterns and seven strains that showed distinct patterns (three noncryptic genital strains, three strains isolated from respiratory tracts, and strain Rd) were selected. The banding patterns obtained on two different days are shown in Fig. 4. The profiles observed were very similar for each strain. The only differences that could be observed were in the staining intensity of some DNA fragments or the absence of weakly staining fragments.

PFGE analysis. Eighty-seven *Haemophilus* strains, including 66 NTHI strains, the 20 genital strains belonging to the cryptic genospecies of *Haemophilus*, and the *H. haemolyticus* strain, were subjected to PFGE. Fingerprints were analyzed and com-

TABLE 2. Frequency of specific IDS-PCR amplification products among 90 *Haemophilus* strains

Fragment size (kb)	No. of strains exhibiting the fragment		
	Cryptic genital strains (<i>n</i> = 20)	Other genital strains (<i>n</i> = 34)	Nongenital strains (<i>n</i> = 36)
Fragments significantly present in cryptic genital strains			
1.9	20 ($P < 0.0000001$) ^a	11	7
0.9	15 ($P = 0.0086$) ^a	14	13
0.7	20 ($P = 0.0000001$) ^a	11	10
0.5	13 ($P = 0.0000019$) ^b	3	4
Fragments significantly absent in cryptic genital strains			
3.3	0 ($P = 0.0023$) ^a	13	14
2.6	0 ($P = 0.0027$) ^b	14	7
1.6	0 ($P = 0.0000025$) ^a	23	21
1.0	0 ($P = 0.0023$) ^a	11	16
0.6	0 ($P = 0.00022$) ^a	13	21
Fragments significantly present in noncryptic genital strains			
2.2	0	19 ($P = 0.00019$) ^a	9
1.4	3	21 ($P = 0.0011$) ^a	11

^a *P* values were calculated by the chi-square test.

^b *P* values were calculated by the two-tailed Fisher's exact test.

FIG. 4. Reproducibility of IDS-PCR. Shown are banding patterns obtained on two different days with 10 *Haemophilus* strains. Lanes 1 to 3, cryptic genital strains C4, C11, and C19; lanes 4 to 6, genital strains G24, G30, and G40; lane 7, strain Rd; lanes 8 to 10, nongenital strains A8, CH8, and CH9; lanes B, reagent blank; lanes M, size markers.

pared with the Taxotron package and were then evaluated using the criteria developed by Tenover et al. (40). The banding patterns obtained by PFGE with 13 *Haemophilus* strains are shown in Fig. 5. The 66 NTHI strains gave patterns consisting of 4 to 15 fragments ranging from 27.5 to 482.8 kb. For these strains, *D* was greater than 0.99. Fifty-nine different restriction patterns were identified for 61 non-epidemiologically-related strains. Epidemiologically related strains gave identical patterns (G24–G25–G26 and G29–G30) (Fig. 5) or patterns that differed by one to three bands (G23–G38 and G6–G7). Only two pairs of non-epidemiologically-related strains that gave distinct patterns with IDS-PCR gave identical patterns with PFGE (G12–G40 and E1–A5). The two non-epidemiologically-related strains that exhibited identical patterns with IDS-PCR, G22 and G23, gave patterns that differed by five bands with PFGE and thus are possibly related according to Tenover's criteria (40). For the 20 genital strains belonging to the cryptic genospecies of *Haemophilus*, PFGE of DNA restricted with *Sma*I was performed several times and no DNA restriction products were observed. As shown in Fig. 5, lane 8, the DNA remained at the top of the gel. The *H. haemolyticus* strain gave a pattern that was distinct from those of all the other strains.

FIG. 5. Banding patterns obtained by PFGE of DNA restricted with *Sma*I for 13 *Haemophilus* strains. Lanes 1 to 3, genital strains G1, G35, and G39; lanes 4 to 7, nongenital strains A4, A7, CH3, and CH6; lane 8, cryptic genital strain C7; lanes 9 and 10, identical profiles of strains G29 and G30; lanes 11 to 13, identical profiles of strains G24, G25, and G26.

DISCUSSION

None of the genotypic methods that have been described for typing of *Haemophilus* strains appear both to be simple and rapid and to provide reproducible and highly discriminatory results. PFGE, which is considered the gold standard, is laborious and time-consuming, and RAPD, which is a rapid and discriminatory technique, lacks reproducibility (15, 17, 36). PCR-based methods such as rep-PCR, which is based on repetitive REP and ERIC sequences, have been developed to offer the advantages of simple, rapid, reproducible, and discriminatory techniques. Nevertheless, for *Haemophilus* typing, REP- and ERIC-PCR are always carried out under low-stringency conditions to allow primer binding. Thus, an annealing temperature between 25 and 40°C (10, 15, 28, 38, 42), 5 mM MgCl₂ (29), or degenerate primers (15) are used. These conditions, which are probably used to compensate for the absence of true REP and ERIC sequences in the *Haemophilus* genome, result in a lack of reproducibility (10, 29).

The aims of our study were to develop a rep-PCR method based on a genuine *Haemophilus*-specific repeated sequence and to validate this technique for typing of *Haemophilus* strains. The uptake signal sequence (USS) is a repeated element present in 1,465 copies on the *H. influenzae* Rd genome (39). rep-PCR using USS-specific primers generated overly complex banding patterns (data not shown) due to the large number of USS copies. This sequence was therefore not selected for rep-PCR. IDS is another repeated element that is present in 14 pairs throughout the *H. influenzae* Rd genome (24, 34). IDS also flanks the *hif* genes, which encode the LKP fimbriae and are absent from strain Rd (3, 35, 43). Thus, IDS-PCR could theoretically generate about 10 DNA fragments with sizes up to 5 kb that would make the analysis possible. We studied 90 strains, including 20 genital *Haemophilus* strains that exhibit a high degree of genetic homogeneity

and are differentiated with difficulty by methods such as MLEE and ribotyping (31, 32). We used a nondegenerate primer that is specific for IDS and high-stringency annealing conditions.

This method was rapid (6 h for amplification and electrophoresis) and easy to perform. It did not require specific and expensive equipment. The banding patterns obtained were simple to read and to compare visually or with the help of a computer program. They were composed of as many as 10 bands with most of the fragments ranging from 150 bp to 2 kb, as expected from the distribution of IDS throughout the *H. influenzae* Rd genome. When IDS-PCR was repeated on 10 *Haemophilus* strains on different days and with two thermocyclers, the resulting patterns were essentially the same, indicating that this technique also had good reproducibility. IDS-PCR is thus more reproducible than RAPD. Its reproducibility is similar to that of ribotyping, for which differences in the staining intensity and the absence of weakly staining DNA fragments can also be observed (32).

IDS-PCR showed a very high discriminatory capacity within the *H. influenzae* species. The 69 NTHI strains generated 65 different fingerprints ($D > 0.99$). Very similar results were obtained by PFGE of DNA restricted with *Sma*I, which is considered the gold standard for *H. influenzae* typing. Sixty-six NTHI strains gave 62 different patterns ($D > 0.99$). These results are in agreement with those obtained by different techniques, showing that NTHI strains are extremely diverse (10, 15, 17, 29, 31, 32, 36). Among the 66 NTHI clinical isolates tested by IDS-PCR, only the epidemiologically related strains (nine strains) and two non-epidemiologically-related strains gave identical or closely related patterns. Similar results were obtained by PFGE. IDS-PCR is consequently a discriminatory method for typing of NTHI strains, as is PFGE, and is considerably less labor-intensive, costly, and time-consuming than PFGE.

IDS-PCR grouped the 20 genital strains belonging to the cryptic genospecies of *Haemophilus* into only 11 closely related fingerprints. Four fragments appeared to be significantly associated with the cryptic genital strains, and six fragments were significantly absent. In this group of strains only, identical banding patterns were obtained for non-epidemiologically-related strains, clustering as many as five strains. The particular homogeneity of this group of strains obtained by this typing method is in agreement with their remarkable genetic homogeneity previously shown and contrasts with the diversity observed in the *H. influenzae* species in general (31, 32). Ribotyping grouped 18 genital strains into seven profiles, two of which included a total of 11 strains (32). MLEE grouped 25 strains into seven electrophoretic types, one of which included 18 strains (31). The discrimination index was thus greater for IDS-PCR within this group ($D = 0.91$) than for ribotyping ($D = 0.81$) or MLEE ($D = 0.49$).

By PFGE, *Sma*I did not restrict the DNA from the 20 cryptic genital strains. Neither did *Apa*I, another endonuclease that is sometimes used to type *Haemophilus* strains by PFGE (23). This could be explained by an inhibition of DNA restriction caused by a methylation of cytosines at the restriction site (5). This is another unusual characteristic of this particular group of genital strains in addition to those previously shown (31, 32).

In conclusion, this study was the first practical application of a rep-PCR assay using a primer designed from the *Haemophi-*

lus-specific repeated sequence IDS as a typing method for nonencapsulated *Haemophilus* strains. Our results suggest that IDS-PCR is a reliable and discriminatory method for typing of NTHI strains, as is PFGE, and is considerably more rapid and less labor-intensive than PFGE. In addition, IDS-PCR is currently the most efficient method for distinguishing strains within the cryptic genospecies of *Haemophilus*.

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