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Genetic Identification of Cryptic Genospecies of *Haemophilus* Causing Urogenital and Neonatal Infections by PCR Using Specific Primers Targeting Genes Coding for 16S rRNA

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Previous genetic analysis of *Haemophilus influenzae* strains isolated from genital and neonatal infections identified a group of biotype IV that constitutes a cryptic genospecies only distantly related to *H. influenzae* and *H. haemolyticus*. Small-subunit rRNA genes of two representative strains of this genital *Haemophilus* genospecies (strains 16N and 2406) were sequenced. The analysis indicated that these strains form a monophyletic unit with *H. haemolyticus* and *H. influenzae* biogroups Influenzae and Aegyptius and are more closely related to *H. haemolyticus* than to *H. influenzae* biogroups Influenzae and Aegyptius. 16S rRNA gene sequences were used to formulate primers for PCR-based identification of cryptic genital *Haemophilus* organisms. A 242-bp fragment was amplified from strains belonging to the genital *Haemophilus* genospecies but not from strains of 12 other *Haemophilus* species, including strains of *H. influenzae* biotype IV sensu stricto.

In recent years, studies in diverse geographic areas have repeatedly identified *Haemophilus influenzae* biotype IV strains as a cause of serious urogenital, neonatal, and mother-infant infections (8, 18, 28). Many of these biotype IV strains from neonatal and urogenital sources have several unusual features. They have characteristic outer membrane protein electrophoretic profiles (22), express a variant P6 outer membrane protein (15), and display increased susceptibility to several quinolones (20). Most of these isolates also express peritrichous fimbriae, adhere more to HeLa cells than to HEp-2 cells, and possess adhesins that do not recognize erythrocyte receptors (23).

The chromosomal genotypes of these isolates are highly divergent from those of all other *H. influenzae* isolates as assessed by multilocus enzyme electrophoresis (16). This result led to the notion that these organisms are a genetically distinct group of bacteria and could be a cryptic genospecies of *Haemophilus*. Subsequent DNA-DNA hybridization experiments showed that these organisms are allied with *H. influenzae* and *H. haemolyticus* and suggested a distant separation of *H. influenzae*, *H. haemolyticus*, and this group of genital *Haemophilus* isolates (17, 19, 21). In addition, these strains have distinctive rRNA gene (rDNA) restriction fragment length polymorphisms that differ from those of *H. haemolyticus* and *H. influenzae* (21).

This group of genital *Haemophilus* isolates can be discriminated from *H. haemolyticus* strains by the hemolytic activity of the latter species and the presence of ornithine decarboxylase in the former but cannot be distinguished from *H. influenzae* isolates sensu stricto. Because these organisms are often recovered from mothers and their neonates with severe infections, convenient genotypic or phenotypic criteria for discriminating between the two groups would be very valuable. A rapid and accurate method for identifying these bacteria from vaginal or gastric fluid cultures would assist obstetricians in formulating appropriate antibiotic therapy.

For this purpose, we sequenced nearly complete 16S rRNA genes of *H. influenzae* biotype IV sensu stricto, *H. influenzae* biotype III, and two representative strains of the genital *Haemophilus* genospecies. These data were combined with previously published sequences to better define the phylogenetic position of the genital *Haemophilus* genospecies within the *Pasteurellaceae* family (6). Specific sequences within the 16S rDNAs of the representative strains of the genital *Haemophilus* genospecies were successfully used to formulate primers to identify these bacteria by PCR.

MATERIALS AND METHODS

Bacterial isolates. Forty-eight *Haemophilus* strains were studied, including 22 reference strains of several human *Haemophilus* species, 7 strains of *H. influenzae*, 1 strain of *H. parainfluenzae*, and 18 strains previously assigned to a cryptic genital *Haemophilus* genospecies on the basis of multilocus enzyme electrophoresis (16, 19) and DNA-DNA hybridization (17, 21) (Tables 1 and 2). Strains were stored at −80°C in Schaedler-vitamin K3 broth (bioMérieux, Marcy l'Etoile, France) containing 10% glycerol and grown on chocolate agar plates (bioMérieux) at 37°C with 10% CO_2.

The phenotypic characteristics of the clinical isolates have been described previously (2, 4, 16, 22, 28). Strains were identified as *Haemophilus* on the basis of colonial morphology, Gram stain, requirement for hemin and NAD, synthesis of porphyrin, and hemolytic activity (10, 11).

During initial steps of isolation, clinical isolates of *H. influenzae* were capsule serotyped (19) and biotyped by conventional methods (11) and four micromethods, including the API 10E and API 20E systems (API-System, Montalieu Vercieu, France), RIM Haemophilus-1/RIM Haemophilus-2 (Ortho Diagnostic Systems, ABL, Austin, Tex.), and the HNID panel (Microscan Division, Baxter Healthcare Corp., West Sacramento, Calif.) (17).

Bacterial DNA preparation. Each strain was subcultured on six chocolate agar plates (15 by 15 cm) for 18 to 24 h at 37°C in 8% CO_2. The cultures were checked visually for purity, harvested in 30 ml of buffer (40 mM Tris, 2 mM EDTA, pH 8.0), and lysed by adding 220 μl of a 25% (wt/vol) aqueous solution of sodium

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TABLE 1. Reference designations and origins of *Haemophilus* sp. strains used to test 16S rDNA-targeted primers able to identify a cryptic genital *Haemophilus* genospecies by PCR

Species	Biogroup or biovar (serovar)	Reference strain designation	Other designation(s)	Origin
Reference strains				
H. actinomycetemcomitans		CIP 52106^T	ATCC 33384, NCTC 9710	Abscess
H. paraphrophilus		CIP 7072^T	ATCC 29241, NCTC 10557	Paronychia
H. aphrophilus		CIP 7073^T	ATCC 33389, NCTC 5906	Unknown
H. haemoglobinophilus		CIP 5388^T	ATCC 19416, NCTC 1659	Prepuce of dog
H. parasuis		CIP 100918^T	ATCC 19417, NCTC 4557	Unknown
H. segnis		CIP 103292^T	NCTC 10977	Dental plaque
H. parainfluenzae		CIP 102513^T	ATCC 33392, NCTC 7857	Septic finger
H. parahaemolyticus		CIP 5686^T	ATCC 10014, NCTC 8479	Pulmonary infection
H. paraphrohaemolyticus		CIP 102512^T	ATCC 29237, NCTC 10670	Sputum
H. influenzae	Aegyptius	CIP 52129^T	ATCC 11116, NCTC 8502	Conjunctiva
H. haemolyticus		NCTC 10659^T	CIP 103290	Sputum
H. haemolyticus		CIP 102348		Sputum
H. influenzae	I(b)	CIP 52152		Unknown
H. influenzae	II(nc)	NCTC 8143^T	ATCC 33391, CIP 102514	Unknown
H. influenzae	III(nc)	CIP 102284		Sputum
H. influenzae	IV(nc)	CIP 5424		Unknown
H. influenzae	IV(d)	CIP 5483		Unknown
H. influenzae	IV(b)	CIP 5494	NCTC 8468	Spinal fluid
H. influenzae	IV(e)	CIP 5484	ATCC 8142, NCTC 8472	Unknown
H. influenzae	IV(d)	CIP 52154		Unknown
H. influenzae	IV(e)	CIP 52155		Unknown
H. influenzae	VI	CIP 102121		Peritoneal fluid
Clinical isolates				
H. influenzae	I	4N		Gastric fluid[a]
H. influenzae	II	1N		Gastric fluid[a]
H. influenzae	II	21N		Gastric fluid[a]
H. influenzae	II	25N		Gastric fluid[a]
H. influenzae	III	13N		Gastric fluid[a]
H. influenzae	III	8N		Gastric fluid[a]
H. influenzae	VI	9N		Gastric fluid[a]
H. parainfluenzae	II	19N		Gastric fluid[a]

[a] Of neonates.

dodecyl sulfate and 30 μl of 1% pronase (Sigma, St. Louis, Mo.) (21). The mixture was incubated overnight at 37°C, and DNA was extracted and purified (3).

Small-subunit rDNA sequencing. One hundred nanograms of DNA was used for PCR to amplify the small-subunit rDNA. Primers corresponding to positions 8 to 28 and 1491 to 1508 of the *Escherichia coli* small-subunit rDNA sequence were used. The procedures for amplification and direct sequencing of PCR products were described previously (24).

Phylogenetic analysis. The phylogenetic data described below were obtained by (i) use of successive alignment and phylogeny procedures and (ii) reinvestigation of deep branching patterns after close relationships had been determined (24). The neighbor-joining method (25) was used for preliminary analysis, and resulting topologies were further investigated by maximum-likelihood and maximum-parsimony analyses. Maximum-likelihood analyses used the fdnaml program rewritten by G. J. Olsen (University of Illinois, Urbana) and compiled on a Hewlett-Packard 700 workstation, and maximum-parsimony analyses were performed with the PAUP program for the Macintosh computer (26). The robustness of each topology was evaluated under maximum parsimony through 100 bootstrap replications (heuristic search). Trees were plotted by using the njplot program for the Macintosh computer developed by M. Gouy (Unité de Recherche Associée 243, Centre National de la Recherche Scientifique, Université Claude Bernard, Lyon, France), which allows the transformation of a formal tree representation (Newick's format) into MacDraw drawings. Only topologies that were found to be similar by all three methods were retained as true trees. Recent theoretical works have demonstrated that similarity of the results obtained by all three methods is a strong indication that the correct phylogeny has been determined (12).

Domains used. Different parts of the small-subunit rDNA sequences were used for Fig. 1. These correspond to positions 6 to 48, 66 to 180, 182 to 426, 450 to 560, 565 to 810, 819 to 976, 992 to 1103, and 1108 to 1419 in the sequence of genital *Haemophilus* strain 16N (accession no. X87978 in the EMBL database).

These domains are shorter than the total length of rDNA sequences obtained. Indeed, nucleotides that are in domains for which a proper alignment cannot be obtained between all of the sequences analyzed should be excluded from phy-

TABLE 2. Reference designations and origins of 18 strains belonging to a previously unrecognized genital *Haemophilus* species[a]

Reference designation	Origin	
	Collection locality	Anatomic site
1610	United States	Blood culture of neonate
911	United States	CSF[b] of neonate
189	United States	Amniotic fluid
421	United States	Blood culture of neonate
1595	United States	Blood culture of neonate
799	United States	Blood culture of neonate
427	United States	Amniotic fluid
422	United States	Blood culture of neonate
3N	France	Gastric fluid of neonate
10N[c]	France	Gastric fluid of neonate
12N	France	Gastric fluid of neonate
15N	France	Gastric fluid of neonate
16N[d]	France	Gastric fluid of neonate
10U	France	Urethral swab (male)
11PS	France	Urethral swab (male)
26E	France	Endouterine samples
PIZ	France	Endocervical swab
2406	France	Vagina

[a] Studied by multilocus enzyme electrophoresis (16, 19) and DNA-DNA hybridization (17, 21).
[b] CSF, cerebrospinal fluid.
[c] Other designation: CIP 103722 or CCUG 31339.
[d] Other designation: CIP 103723 or CCUG 31340.

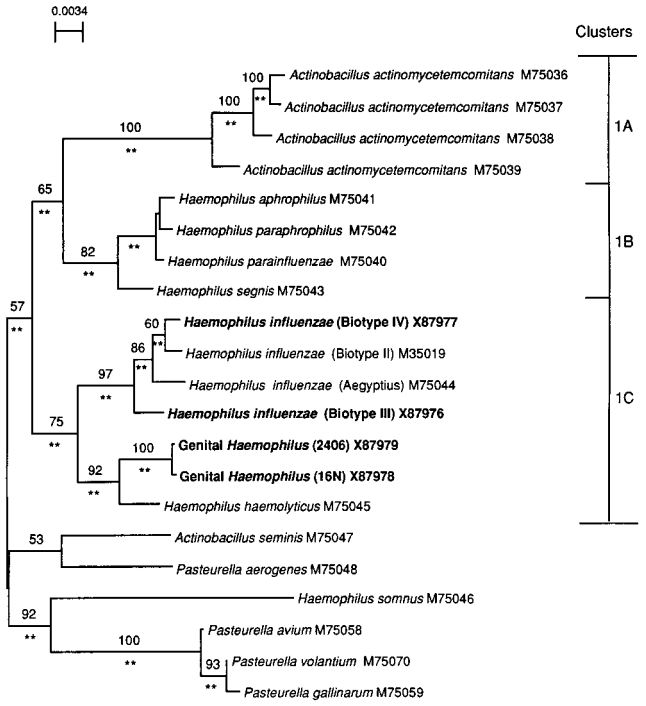

FIG. 1. Phylogenetic position of the genital *Haemophilus* genospecies within a subset of *Pasteurellaceae* restricted to species representative of cluster 1 and the most related species (6). The topology shown is an unrooted tree obtained with the neighbor-joining method. The double asterisks indicate branches that were also found by maximum-likelihood analysis ($P < 0.01$). Values above the lines (only values greater than 50% are shown) show branches also found in the most parsimonious tree and indicate how these branches were supported by bootstrap analysis (heuristic search, 100 replications). The scale bar shows accumulated changes per nucleotide. Strains whose sequences were determined in this study are in boldface. The domains used to prepare this tree are indicated in Materials and Methods.

logenetic analyses. It is also necessary to exclude positions that can be well aligned but are mutation hot spots. For such nucleotides, homoplasies predominate over the true phylogenetic signal. A robust phylogenetic analysis is therefore obtained only when these two types of positions are excluded from the analysis.

The similarity values shown in Table 3 were calculated by using the entire length of the rDNA sequenced.

Amplification of 16S rDNA. We tested whether PCR amplification with a selection of primers deduced from the sequence data is able to differentiate cryptic genital *Haemophilus* isolates from members of other *Haemophilus* species. The PCR mixture contained 100 ng of genomic DNA, each primer at 0.5 μM, each deoxynucleoside triphosphate at 100 μM, and 1.5 U of *Taq* DNA polymerase (Appligene, Pleasanton, Calif.) in a final volume of 100 μl. The amplification protocol included 22 cycles of PCR and consisted of a denaturing step at 95°C for 1 min, annealing at 50°C for 2 min, and extension at 72°C for 2 min. The first denaturing step was conducted at 95°C for 2.5 min, and the final elongation step was done at 72°C for 10 min. The amplified fragment was visualized by horizontal gel electrophoresis for 3 h at a constant 100 V in 1.4% agarose (type II; Sigma).

To simplify and reduce the time required for sample preparation, the target DNA was also prepared from boiled suspensions of bacteria. To validate the method, the results were compared with those obtained after DNA extraction and purification. For this purpose, cells were scraped from chocolate agar plates and resuspended in 1 ml of TE buffer (10 mM Tris HCl, 1 mM EDTA [pH 8.0]). The turbidity of the suspension was measured at 600 nm and adjusted to an optical density of 1.5. Each suspension of bacteria was heated at 100°C for 10 min. After centrifugation for 5 min at 15,000 × *g*, 5 μl of the supernatant was used as template DNA for PCR amplification.

Nucleotide sequence accession numbers. Sequences have been deposited in the EMBL sequence database under accession numbers X87976 (*H. influenzae* biotype III, CCUG7585), X87977 (*H. influenzae* biotype IV, CIP 5483), X87978 (*H. influenzae* 16N, CIP 103723), and X87979 (*H. influenzae* 2406).

RESULTS

Phylogenetic relationships among strains of *H. influenzae* and related species. General phylogenetic analyses based on rDNA sequences confirmed that all of the *Haemophilus* species studied belong to the gamma 3 branch (6, 24) of the phylum *Proteobacteria*, more precisely, to the family *Pasteurellaceae* (data not shown). More-detailed analyses were then restricted to species representative of cluster 1 and the most closely related species within this family as previously described (6). As shown in Fig. 1, *H. haemolyticus* and all strains of *H. influenzae* biogroups Influenzae and Aegyptius were grouped in a monophyletic unit corresponding to cluster 1C, a result supported by all three phylogenetic methods and a bootstrap value of 75%. Genital *Haemophilus* strains 2406 and 16N were more closely related to *H. haemolyticus* than to *H. influenzae* biogroups Influenzae and Aegyptius. These two strains had an rDNA sequence similarity of 99.8%, whereas similarities lower than 97.9% were found with all other species of cluster 1C (Table 3).

Selection of specific primers. We selected two primers for PCR to identify the strains belonging to the cryptic genital *Haemophilus* genospecies. The primers were formulated on the basis of comparison of 77 small-subunit aligned rDNA sequences belonging to the family *Pasteurellaceae*. The two regions chosen are located in two variable regions of the 16S rDNA sequences. Table 4 shows these two regions for 21 aligned small-subunit rDNA sequences and the sequences of two 20-nucleotide primers. It was not possible to select a primer specific for the genital *Haemophilus* genospecies in the 5′ end of 16S rDNA sequences. However, a sequence was chosen that hybridized with neither *H. influenzae* biotype IV sensu stricto nor *H. haemolyticus*, the two species with which the genital *Haemophilus* genospecies can be readily confused. Primer 1 corresponded to the sense strand at positions 164 to 183 of the genital *Haemophilus* strain 16N sequence available under accession no. X87978. In the 3′ end, a primer specific for the genital *Haemophilus* isolates corresponded to the antisense strand at positions 445 to 426 (Table 4).

Identification of the cryptic genital *Haemophilus* genospecies by PCR. The two selected primers were used to test 48 strains of various *Haemophilus* species of medical interest by PCR (Tables 1 and 2).

TABLE 3. Similarity matrix based on entire small-subunit rDNA sequences

Organism	Accession no.[a]	% Similarity with: Genital *Haemophilus* strain 2406	*H. haemolyticus*	*H. influenzae* biotype II	*H. influenzae* biotype III	*H. influenzae* biotype IV	*H. influenzae* biogroup Aegyptius
Genital *Haemophilus* strain 16N	X87978	99.8	97.9	95.7	96.6	96.4	95.8
Genital *Haemophilus* strain 2406	X87979		97.8	95.7	96.6	96.4	95.8
H. haemolyticus	M75045			95.7	95.7	96.0	96.1
H. influenzae biotype II	M35019				99.1	99.2	99.3
H. influenzae biotype III	X87976					98.4	98.9
H. influenzae biotype IV	X87977						99.1

[a] Sequences are available in the EMBL database.

TABLE 4. Alignment of sequences in the two 16S rDNA regions in which two 20-nucleotide primers were selected for specific amplification of genital *Haemophilus* isolates[a]

Organism	Accession no.	5′ end of 16S rDNA target sequence					3′ end of 16S rDNA target sequence				
Haemophilus spp.		5′ ATTAAAGT	G	TGGGACCTTC	G 3′[b]				3′ CCTACACACG	ATTATCATGT 5′[c]	
Genital *Haemophilus* strain 16N	X87978	ATATCGANAG	ATTAAAGT*G	TGGGACCTTC	GGGCCACATG	CCATAAGATG	TTCTTTCGGT	ATTGAGGAAG	GGATGTGTGC	TAATAGTACA	NNTCATTGAC
Genital *Haemophilus* strain 2406	X87979	-------A--	----------	----------	----------	----------	----------	----------	----------	----------	CG--------
H. haemolyticus	M75045	G-G----G--	-CG-------	C------GCA	A----G----	----G-----	----------	N---------	-AG---A--T	------C-T-	CA-T------
H. influenzae biotype II	M35019	T-----GA--	--G-------	C-----TGAG	A----G----	-----G----	----------	----------	-TTGA----T	------C---	TCAA------
H. influenzae biotype III	X87976	-------A--	----------	----------	----------	-----G----	----------	----------	-TTGA----T	------C---	TCAA------
H. influenzae biotype IV	X87977	T-----GA--	--G-------	C-----TGAG	A----G----	-----G----	----------	----------	-TTGA----T	----------	TCAA------
H. influenzae biogroup Aegyptius	M75044	G-G----G--	-CG-------	C-----TGAG	A----G----	-----G----	----------	----------	-TTGA----T	------C---	TCAA------
H. aphrophilus	M75041	GA----GG--	--G-------	----------	----------	-----G----	----------	GAC-------	-TTGT----T	------CG--	ACAA------
H. paraphrophilus	M75042	GN-----G--	--G-------	---------N	----------	-----G----	----------	GAC-------	-TTGT----T	G-----C---	ACAA------
H. parainfluenzae	M75040	GA-----G--	--G-------	----------	----------	-----G----	----------	GAC-------	-TTGT----T	------CG--	ACAA------
H. segnis	M75043	GAG----G--	-CG-------	C------GCA	A----G----	-----G----	----------	NAN-------	-CG--ATGTT	------GCAT	CACG------
H. somnus	M75046	-GG--TNAG-	-C-----GG-	------TNAG	A------C-N	----TT----	----------	GA--------	-CGAT-AGTT	---G--ATT-	AT-G------
Actinobacillus spp.											
A. actinomycetemcomitans	M75039	GA----GG--	--G-------	----------	----------	-----GA---	----------	GA--------	-TTG-----T	------C-TG	CCAA------
A. actinomycetemcomitans	M75038	GAG---GG--	-CG-------	C--------A	-----G----	----G-----	----------	GA--------	-TTG-----T	------C-TG	CCAAC-----
A. actinomycetemcomitans	M75037	GAG---GG--	-CG-------	C-----T--N	T----G----	----G-----	----------	----------	-TTG-----T	------C-TG	CCAA------
A. actinomycetemcomitans	M75036	GGG---GG--	-CG-------	C-----T--N	T----G----	----G-----	----------	----------	-TTG-----T	------C-TG	CCAA------
A. seminis	M75047	G-----GG--	--G----G--	------TGAA	-------C--	----------	----------	NG--------	-TGATGAA-T	G--G---TTC	ATCA------
Pasteurella spp.											
P. aerogenes	M75048	G-G----G--	-CG----A-C	C------GCN	A----GGT--	----------	----------	GA--------	-CGGT-AGTT	------GCT-	ATCG------
P. avium	M75058	G------G--	-------G--	------T--T	TA-----C--	----------	----------	GG--------	-TTG-A---T	------CG-T	CTAAT-----
P. volantium	M75070	GA-----A--	-------G--	------T--T	TA-----C--	----------	----------	GG--------	-CGGTA---T	------Y--T	AY-GT-----
P. gallinarum	M75059	G------A--	-------G--	-----NT--T	TA-----C--	----------	----------	GG--------	-CGGTA---T	-N----CG-T	AT-GT-----

[a] Species shown are those of Fig. 1. The sequence of the genital *Haemophilus* genospecies is used as a reference. Positions identical to those of the first sequence are indicated by dashes. The asterisk shows the deletion necessary to maximize homology.
[b] Primer 1.
[c] Primer 2.

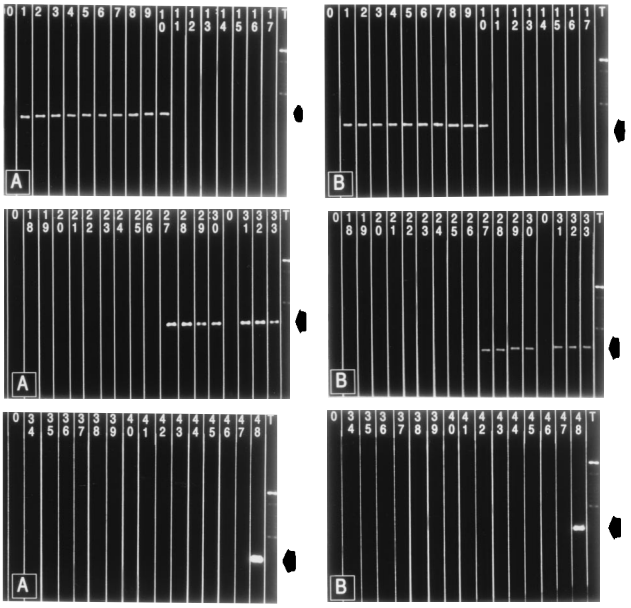

FIG. 2. Identification of a genital *Haemophilus* genospecies by PCR. A 242-nucleotide fragment (◀) from each of the 18 strains belonging to this genospecies was amplified. Strains: 16N (lane 1), 422 (lane 2), 2406 (lane 3), 427 (lane 4), 799 (lane 5), 1595 (lane 6), 421 (lane 7), 189 (lane 8), 911 (lane 9), 1610 (lane 10), 15N (lane 27), 12N (lane 28), 10N (lane 29), 3N (lane 30), PIZ (lane 31), 26E (lane 32), 10U (lane 33), and 11 PS (lane 48). No specific fragments were amplified for 6 *H. influenzae* biotype IV sensu stricto strains (CIP 52155 [lane 11], CIP 52154 [lane 12], CIP 5484 [lane 13], CIP 5494 [lane 14], CIP 5483 [lane 15], and CIP 5424 [lane 16]), seven clinical isolates of *H. influenzae* (8N [lane 18], 4N [lane 19], 1N [lane 20], 25N [lane 36], 21N [lane 37], 13N [lane 39], and 9N [lane 40]), one clinical isolate of *H. parainfluenzae* (19N [lane 38]), 5 reference strains of *H. influenzae* (NCTC 8143^T [lane 17], CIP 102121 [lane 35], CIP 102284 [lane 42], CIP 52152 [lane 47], and biogroup Aegyptius strain CIP 52129^T [lane 34]), and 11 reference strains of various *Haemophilus* species (*H. parasuis* CIP 100918^T [lane 21], *H. parainfluenzae* CIP 102513^T [lane 21], *H. parahaemolyticus* CIP 5686^T [lane 23], *H. actinomycetemcomitans* CIP 52106^T [lane 24], *H. paraphrohaemolyticus* CIP 102512^T [lane 25], *H. haemolyticus* NCTC 10659^T [lane 26], *H. aphrophilus* CIP 7073^T [lane 41], *H. paraphrophilus* CIP 7072^T [lane 43], *H. haemoglobinophilus* CIP 5388^T [lane 44], *H. segnis* CIP 103292^T [lane 45], and *H. haemolyticus* CIP 102348^T [lane 46]). The results were comparable when target DNA was extracted and purified (A) and when target DNA was prepared from boiled suspensions of bacteria (B). See Tables 1 and 2 for origins of isolates.

A 242-bp fragment of the 18 strains belonging to the genital *Haemophilus* genospecies was amplified. In contrast, an amplified fragment was not obtained with the other 11 species tested (Fig. 2). This PCR assay was able to differentiate the strains of the genital *Haemophilus* genospecies from 18 clinical and reference strains of various *H. influenzae* biotypes, including 6 strains of biotype IV sensu stricto, and from two strains of *H. haemolyticus* (Fig. 2).

DISCUSSION

Genital, perinatal, and neonatal infections due to *Haemophilus* strains were first described in the early 1900s (5, 14, 27). The number of reported cases has increased in the last 20 years (7, 13, 14, 22, 28). Although specific tropism of some *H. influenzae* strains for the genital tract has been suggested on the basis of biotyping and serotyping studies (1, 18, 22, 28), analysis of outer membrane protein patterns and genetic characterization of isolates do not support a general concept of specific genital strains. The exception is a very homogeneous group of genital strains, usually assigned to *H. influenzae* biotype IV, which share singular phenotypic and genotypic characteristics (15, 17, 19, 20, 21, 22, 23).

In the 1980s, characterization of *H. influenzae* strains by multilocus enzyme electrophoresis identified a homogeneous group of biotype IV organisms. Most of these bacteria, originating from the genital tract and responsible for serious obstetric and neonatal infections, were highly divergent from the other *H. influenzae* strains (16, 19). Genomic DNA-DNA hybridizations demonstrated that these biotype IV isolates share far less than 70% overall genomic similarity with *H. influenzae* (17, 19), which means that this group of genital *Haemophilus* isolates constitutes a genospecies only distantly related to *H. influenzae* and *H. haemolyticus* (21).

Our present experiments, based on comparisons of nearly complete small-subunit rDNA sequences, demonstrate that two representative strains of this genital *Haemophilus* genospecies (strains 2406 and 16N) form a monophyletic group related more to *H. haemolyticus* than to *H. influenzae* biogroups Influenzae and Aegyptius. This result is in agreement with the high homology values recorded among the genomic DNAs of these strains (21).

There are no phenotypic characters easily usable by clinical microbiology laboratories to differentiate this cryptic group of genital *Haemophilus* bacteria reliably from isolates of *H. influenzae*. As previously shown, the two groups cannot be accurately differentiated by conventional or commercial methods commonly used for identification and biotyping of *Haemophilus* strains (17). We tested 75 additional biochemical characteristics but did not find a useful test for differentiation of this genital *Haemophilus* genospecies from *H. influenzae* sensu stricto (data not shown). In addition, some strains of this genital *Haemophilus* genospecies are sometimes identified as *H. parainfluenzae* because of a positive sucrose test (17). PCR with the two specific primers targeting 16S rDNA proposed in this work accurately differentiates strains belonging to the genital *Haemophilus* genospecies from the other *Haemophilus* species. In addition, this technique is able to discriminate *H. influenzae* biotype IV sensu stricto from the cryptic genital *Haemophilus* group. Therefore, this molecular method is a valuable and efficient technique which can be used by clinical microbiologists.

When a culture of a genital or neonatal sample is positive for gram-negative rods belonging to the genus *Haemophilus*, identification by PCR and phenotypic characterization with a rapid commercial system may be conducted in parallel. Complete identification including the genital *Haemophilus* genospecies is thereby possible in less than 6 h. We suggest that this procedure be used to characterize all *Haemophilus* strains isolated from genital tract sources and neonates because some strains of the genital *Haemophilus* genospecies may be misidentified as other *Haemophilus* species (17) or, as recently shown, as an *H. influenzae* biotype other than IV (9).

PCR is a rapid and suitable identification method, but it may not be within the diagnostic scope of some routine laboratories. Therefore, the specific sequence described here (primer 2 in Table 4) may be the basis for the development of a probe to be used, for example, in a DNA immunoassay.

In conclusion, small-subunit rDNA sequencing indicates that some *Haemophilus* strains isolated from genital and neonatal infections belong to a monophyletic unit including *H. influenzae* biogroups Influenzae and Aegyptius and *H. haemolyticus* and are more closely related to *H. haemolyticus*. Two primers deduced from the 16S rDNA sequences can be used by clinical microbiologists to identify this genital *Haemophilus* genospecies efficiently by PCR.

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