Urogenital, maternal and neonatal isolates of *Haemophilus influenzae*: identification of unusually virulent serologically non-typable clone families and evidence for a new *Haemophilus* species

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A collection of 117 strains of *Haemophilus influenzae*, including 112 non-typable isolates recovered predominantly in the USA and France from genital, obstetric and neonatal sources, was characterized by the electrophoretic mobilities of 10 metabolic enzymes. Eighty-six distinctive multilocus chromosomal genotypes (electrophoretic types, ETs) were distinguished on the basis of allele profiles at the enzyme loci. Isolates of five allied biotype IV ETs were highly divergent from all other strains and hybridization of chromosomal DNA revealed that they undoubtedly represent a previously unrecognized species of *Haemophilus*. Isolates representing these ETs were recovered predominantly from obstetric infections and serious neonatal diseases and apparently possess specific tropism for the genital tract. Strains of these five ETs were present in samples from both the USA and France, but only in the USA did they cause bacteraemia and meningitis, an occurrence which probably reflects differences in patient management between the two countries. Although strains assigned to *H. influenzae* (*sensu stricto*) were strongly polymorphic in multilocus enzyme genotype, 69% of isolates recovered from patients with meningitis and/or septicaemia were assigned to only two clone families, a result suggesting that some serologically non-typable strains of *H. influenzae* originating from the genital tract are unusually virulent.

Introduction

Genital and mother–infant infections, neonatal septicaemia and meningitis episodes caused by *Haemophilus influenzae* were reported with increased frequency in the 1980s (Wallace *et al.*, 1983; Kleiman *et al.*, 1983; Friesen & Cho, 1986; Quentin *et al.*, 1987). Unlike strains recovered from invasive episodes in children occurring after the first month of life, those isolated from neonates are usually non-typable. Currently, 5–8% of cases of invasive neonatal diseases in some states of the USA are caused by non-encapsulated *H. influenzae* (Wallace *et al.*, 1983; Campagnone & Singer, 1986), and these infections are associated with significant morbidity and mortality.

In striking contrast, in a six-year study in France, only a single case of *H. influenzae* neonatal meningitis was recorded (caused by a serotype b strain) and no episodes of neonatal or maternal sepsis were recorded (Quentin *et al.*, 1989).

Characterization of outer-membrane proteins of genital and neonatal isolates from France and the USA by SDS-PAGE revealed two phenotypic populations of *H. influenzae*. One population comprised *H. influenzae* strains of all biotypes and appeared quite similar to the heterogeneous population isolated from upper respiratory tract infection. A second population was composed of *H. influenzae* strains of biotype IV, and was relatively homogeneous in outer-membrane protein type. Comparison of *H. influenzae* strains isolated from patients in these countries did not identify major phenotypic

Abbreviation: ET, electrophoretic type.

differences that would account for the more favourable outcomes observed in French patients (Quentin *et al.*, 1989).

In addition, in the course of a study of the genetic relationships of serologically non-typable and serotype b *H. influenzae* isolates by multilocus enzyme electrophoresis, four unencapsulated strains were identified which were genetically highly divergent from all others examined and were hypothesized to be specifically distinct (Musser *et al.*, 1986a). These strains were closely similar in chromosomal genome, were of biotype IV, and had originated from cases of neonatal bacteraemia or obstetric infections.

The objectives of the research reported here were to examine further the molecular epidemiology, genetic diversity and relatedness of isolates of *H. influenzae* from neonatal, maternal and urogenital sources by assaying allelic variation in a sample of chromosomal enzyme loci. Specifically, our goals were to (i) assess the frequency of occurrence of strains with highly divergent genotypes, (ii) ascertain the genetic relationship of these divergent genotypes with respect to the species *H. influenzae*, and (iii) examine associations of distinctive bacterial genotypes and systemic infections.

Methods

Bacterial isolates. A collection of 117 strains of *H. influenzae*, isolated in the 1970s and 1980s from genital and neonatal infections, was used. Eighty isolates were recovered from patients in the Bretonneau University Hospital in Tours, France; 37 cultures originated from North America, of which 36 were from the USA and one from Canada. The anatomic origins of the isolates are listed in Table 1. Some of the isolates have been described previously (Wallace *et al.*, 1983; Kleiman *et al.*, 1983; Campognone & Singer, 1986; Musser *et al.*, 1986a; Quentin *et al.*, 1987, 1989).

Serotyping. Isolates from France and Canada were serotyped upon isolation with co-agglutination reagents (Phadebact-*Haemophilus* test; Pharmacia) and controlled by slide agglutination and/or counter-current immunoelectrophoresis with commercial type-specific rabbit antisera prepared against *H. influenzae* capsular types a to f (Difco). Isolates from the USA were serotyped by slide agglutination with type-specific commercial antisera (Difco), or with type-specific antisera prepared by the Centers for Disease Control, Atlanta, Georgia, USA (Wallace *et al.*, 1983). Most isolates from the USA were also serotyped by counter-current immunoelectrophoresis with uncentrifuged culture broth and type-specific antisera (type b, Hyland Diagnostics, Deerfield, Illinois, USA; types a, c, d, e and f, Burroughs-Wellcome) (Wallace *et al.*, 1983).

Biotyping. All isolates from Canada and France were biotyped with the API 20E system (API System, Montalieu Vercieu, France), as described by Holmes *et al.* (1982). Isolates from the USA were tested for urease activity, indole production and ornithine decarboxylase activity (Kilian, 1976, 1980) with the Minitest system (BBL Microbiology Systems), as described previously (Wallace *et al.*, 1983).

Enzyme electrophoretic analysis. Each isolate was grown overnight at 37 °C in 150 ml brain heart infusion broth supplemented with NAD and haemin, each at 2 µg ml⁻¹. Cells were harvested by centrifugation,

Table 1. Collection locality and anatomic site of recovery for 117 genital and neonatal isolates of *H. influenzae*

Anatomic site	Country		Total
	France	USA	
Gastric fluid of neonates heavily colonized	19	5	24
Placenta		1	1
Cerebrospinal fluid of neonates	1	3*	4
Blood of neonates†		18	18
Blood of mother‡		4	4
Curettage product during septic miscarriages	4		4
Endouterine samples	24		24
Peritoneal fluid during salpingitis	5		5
Surgically obtained pus:			
Bartholinitis	5	1	6
Orchepididymitis	1		1
Rectal abscess (male)		1	1
Scrotal abscess		1	1
Vaginal and endocervical swabs	16	3	19
Urethral swabs (male)	2		2
Sperm culture	3		3
Total	80	37	117

* One strain was from Canada.

† In two cases, blood cultures of mother were also positive.

‡ With negative blood cultures in the neonates.

suspended in 2 ml 50 mM-Tris/5 mM-EDTA (pH 7.5), and sonicated (Branson Sonifier Cell Disruptor, model 200, with microtip) for 30 s at 50% pulse, with ice-water cooling. After centrifugation at 20000 g for 20 min at 4 °C, the clear supernatant (lysate) was stored at -70 °C.

The techniques of horizontal starch-gel electrophoresis and the demonstration of specific enzyme activity were similar to those described by Selander *et al.* (1986). The following 10 enzymes were assayed: phosphoglucose isomerase (PGI), NAD-dependent malate dehydrogenase (MDH), glucose-6-phosphate dehydrogenase (G6P), glutamic oxaloacetic transaminase (GOT), adenylate kinase (ADK), 6-phosphogluconate dehydrogenase (6PG), leucylalanine peptidase-1 (PEP), leucine aminopeptidase (LAP), phosphoglucomutase (PGM), and glutamate dehydrogenase (GLD).

For each enzyme, distinctive mobility variants were designated as electromorphs and numbered in order of decreasing rate of anodal migration. Electromorphs of an enzyme were equated with alleles at the corresponding structural gene loci. Because all isolates showed activity for all 10 enzymes, the corresponding structural gene loci are presumed to be located on the chromosome rather than on plasmids.

Each isolate was characterized by its combination of alleles at the 10 enzyme loci, and distinctive profiles of electromorphs, corresponding to unique multilocus genotypes, were designated as electrophoretic types (ETs) (Selander *et al.*, 1986). [The numerical designations of alleles and ETs used in the present study do not correspond to those used by Musser *et al.* (1985, 1986a, 1988b).]

Strains representing ETs 81 to 86 were also examined for electrophoretic variation of NADP-dependent malic enzyme, leucylalanine peptidase-2, fumarase, glyceraldehyde-3-phosphate dehydrogenase, nucleoside phosphorylase, catalase and carbamylate kinase.

Genetic diversity at an enzyme locus (*h*) among either ETs or isolates was calculated from the allele frequencies as $h = (1 - \sum x_i^2) / [n(n-1)]$, where x_i is the frequency of the *i*th allele and *n* is the number of ETs (Selander *et al.*, 1986). Mean genetic diversity per locus (*H*) is the arithmetic average of *h* values for all loci. Genetic distance (*D*) between

pairs of ETs was calculated as the number of loci at which dissimilar alleles were represented (mismatches), and clustering of ETs from a matrix of pairwise coefficients of genetic distance was performed by the average-linkage method (Selander *et al.*, 1986).

DNA-DNA hybridization. Nine strains representative of ETs 81–85 and the *H. influenzae* type strain NCTC 8143^T (National Collection of Type Cultures, Colindale, London, UK) were each cultured on six 15 cm × 15 cm plates of chocolate agar medium (bioMérieux, Charbonnières les Bains, France). After incubation for 18–24 h at 37 °C in 8% CO₂ and a visual check for purity, bacteria were harvested in 30 ml buffer (40 mM-Tris/2 mM-EDTA, pH 8). Cells were lysed by adding 220 µl of a 25% (w/v) aqueous solution of sodium dodecyl sulphate and 30 µl of 2% (w/v) pronase (Sigma). The mixture was incubated at 37 °C overnight to achieve cell lysis. DNA was extracted and purified according to Brenner *et al.* (1982). Unlabelled DNA was sheared by sonication and dialysed overnight against 0.042 M-NaCl, then stored at 4 °C. Native DNA of strain NCTC 8143^T was labelled *in vitro* by nick translation (Brenner *et al.*, 1982) with [³H]cytidine (Amersham). For DNA-DNA hybridization experiments, the S1 nuclease method using DEAE-cellulose filters (2.4 cm DE 81 filter, Whatman) was performed as described by Popoff & Coynault (1980). Labelled DNA (15 ng) and unlabelled DNA (90 µg) were mixed in a solution containing 0.042 M-NaCl to a final volume of 0.5 ml, heat-denatured for 4 min at 100 °C, then incubated at 60 °C for 16 h.

The following values were determined: the relative binding (%) of DNA at 60 °C; the temperature at which 50% of the reassociated DNA became hydrolysable by the S1 enzyme (*T_m*) (Crosa *et al.*, 1973); and the difference (ΔT_m) between homo- and heteroduplex *T_m* values, expressing the decrease of heteroduplex thermal stability.

Results

Genetic diversity of isolates

There were 112 serologically non-typable isolates, two isolates of serotype a and three isolates of serotype b. All 10 enzymes assayed were polymorphic for five to fourteen electrophoretically demonstrable variants, and the mean number of alleles per locus was 7.3. By comparing the allele profiles of isolates assayed for all 10 enzymes, 86 distinctive multilocus combinations or ETs were identified, among which mean genetic diversity per locus (*H*) was 0.649 (variance 0.014) (Table 2). Thirteen of these ETs were represented by multiple isolates (range 2 to 18).

Genetic relationships among the 86 ETs are represented in the dendrogram shown in Fig. 1. The smallest observed genetic distance (0.10) corresponds to a single-locus difference between ETs, and the largest distance (0.84) corresponds to differences at 9 of the 10 loci assayed.

At a genetic distance of 0.60, there were 10 clusters or lineages of ETs. ET 1 to ET 80 diverge from ET 81 to ET 86 at a genetic distance of 0.84. Among the first group of ETs (ETs 1 to 80) there is a major cluster (cluster A in Fig. 1) of 69 ETs which diverges from ETs 70 to 80 at a genetic distance of 0.62. Strains assigned to cluster A were of various biotypes: 8 isolates of biotype I, 44

Table 2. Genetic diversity (*h*) at 10 enzyme loci in 86 ETs of *H. influenzae* isolated from genital and neonatal infections

Enzyme locus*	No. of alleles	Genetic diversity (<i>h</i>)
PGI	14	0.858
MDH	7	0.715
G6P	8	0.784
GOT	5	0.671
ADK	5	0.496
6PG	7	0.546
PEP	7	0.734
LAP	7	0.590
PGM	5	0.630
GLD	8	0.471
Mean	7.3	0.649

* See Methods for explanation of abbreviations.

isolates of biotype II, 18 isolates of biotype III, 1 isolate of biotype IV, 6 isolates of biotype V, and 4 isolates of biotype VI. Isolates assigned to the various biotypes were distributed essentially randomly throughout cluster A. Isolates assigned to ETs 70 to 80 were also heterogeneous in biotype.

The most genetically divergent strains were assigned to ETs 81 to 86. Among this group of 6 ETs there is a major cluster (cluster B in Fig. 1) of 5 ETs (ETs 81 to 85) that were represented by 23 serologically non-typable isolates, all of which were of biotype IV. Seven of the isolates were recovered from patients in France, 15 isolates were cultured from individuals in the USA, and one isolate was recovered from a neonate in Canada. Analysis of these isolates for electrophoretic variation at seven other enzyme-encoding loci revealed little additional diversity, a result confirming the conclusion that this group is a closely allied cluster of genotypes.

Genetic variation in relation to biotype

Genetic diversity among ETs of each biotype is presented in Table 3, with the exception of biotypes V and VI, which are omitted because of small sample sizes. Mean genetic diversity per locus among ETs of each biotype varied from 0.263 in the case of biotype IV to 0.544 for biotype II, 0.588 for biotype I and 0.594 for biotype III. Thus, the biotype IV isolates were a genetically allied population. In addition, 23 of 25 (92%) biotype IV isolates represented 5 ETs (ETs 81 to 85) clustering within the most genetically divergent group, and 18 of them (78%) were assigned to a single multilocus genotype (ET 81). Nine strains representing ETs assigned to this cluster (five strains of ET 81 and one strain each of ETs 82 to 85) were further characterized by

Fig. 1. Genetic relationships among 86 ETs of urogenital, maternal and neonatal *H. influenzae*. The dendrogram was generated by the average-linkage method of clustering from a matrix of coefficients of pairwise genetic distance (Selander *et al.*, 1986) based on 10 enzyme loci. At a genetic distance of 0.60, there are 10 clusters or lineages of ETs. ETs 1 to 80 correspond to *H. influenzae* (*sensu stricto*) in which two clusters (ETs 37 to 44 and 65 to 69), comprising 69% of the strains recovered from patients with septicaemia and meningitis (○), were identified. Isolates assigned to ETs 81 to 85 (cluster B) represent the putative new species of *Haemophilus*. Most of the strains (18/23) and eight of the nine invasive isolates (●) of this new species were assigned to ET 81.

Table 3. Genetic diversity per locus (*h*) in *H. influenzae* isolates cultured from genital and neonatal infections, classified by biotype

Biotype	No. of isolates	No. of ETs	Mean no. of alleles	Mean genetic diversity
I	12	12	3.4	0.588
II	48	39	5.4	0.544
III	22	18	4.4	0.594
IV	25	7	3.1	0.263

genomic DNA-DNA hybridization experiments. Estimates of the level of DNA relatedness of each isolate to the type strain of *H. influenzae* are shown in Table 4. Strains of biotype IV assigned to ETs 81 to 85 were 52 to 68% (median = 60%) related to the type strain, with ΔT_m values between 6.5 and 8.3 °C (median = 6.9 °C). Hence, according to the generally accepted species criteria (Wayne *et al.*, 1987), biotype IV strains of ETs 81 to 85 are incorrectly classified as *H. influenzae* and represent a previously unrecognized species.

Genetic variation of *H. influenzae* species (*sensu stricto*) (ETs 1 to 80) in relation to clinical episode

Strains responsible for upper genital infections (endometritis, pelvic inflammatory diseases and Bartholin's gland abscesses) did not cluster into a single phylogenetic line. The genetic diversity per locus (0.594) and the frequency of alleles at the 10 enzyme loci among isolates recovered from these diseases were similar to those values recorded for the total sample, indicating that each of these types of disease was caused by strains belonging to a wide variety of chromosomal enzyme genotypes.

In contrast, 9 of the 13 non-serotypable strains isolated from cases of neonatal meningitis and/or mother-infant septicaemia were assigned to only two clusters of enzyme genotypes. The first cluster was composed of 8 strains in 8 ETs diverging at a genetic distance of 0.40 (ETs 37 to 44, Fig. 1) and comprised 5 invasive isolates. The second cluster contained 5 isolates representing 5 ETs diverging at a genetic distance of 0.45 (ETs 65 to 69), of which 4 isolates caused invasive disease episodes. Comparison of the allele frequencies of these 13 strains to those of strains isolated from heavily colonized neonates with no evidence of systemic infection (16 isolates assigned to 15 ETs distributed throughout the dendrogram in an apparently random fashion) revealed that these two populations differed significantly in the frequency of occurrence of certain alleles of MDH and PEP. Most notably, 10 of the 13 strains isolated from systemic infections possessed the 4 allele of MDH (Table 5).

Genetic variation of strains assigned to ETs 81 to 85 in relation to clinical episode

Of 23 strains belonging to this cluster, five of the French isolates were recovered from the gastric aspirates of heavily colonized neonates (Quentin *et al.*, 1987) and one strain each was recovered from women with urethritis and endometritis. The USA isolates, from patients in a total of eight states, were cultured from cerebrospinal fluid (2 isolates), blood (6), amniotic fluid (3), placenta (1), scrotum (1), and one each from the cervix and vagina of women with post-partum sepsis. The single strain

Table 4. Level of DNA relatedness between *H. influenzae* NCTC 8143^T (the type strain) and nine biotype IV strains belonging to the putative cryptic species of *Haemophilus* isolated from neonatal and genital infections

Source of unlabelled DNA	Biotype	Relative binding to NCTC 8143 ^T (%)	<i>T_m</i>	ΔT_m
Salmon testes		5.6	—	—
NCTC 8143 ^T (1st determination)	II	100	87.0	—
NCTC 8143 ^T (2nd determination)	II	100	87.3	—
Strain 3N (ET 81)	IV	63	80.1	6.9
Strain 10N (ET 81)	IV	68	80.5	6.5
Strain 16N (ET 81)	IV	67	80.2	6.8
Strain 101 (ET 81)	IV	60	80.2	6.8
Strain 12N (ET 81)	IV	55	80.8	6.5
Strain 15N (ET 82)	IV	52	80.0	7.3
Strain 427 (ET 83)	IV	67	80.8	6.5
Strain 1610 (ET 84)	IV	58	79.4	7.6
Strain 26E (ET 85)	IV	60	79.0	8.3
Median		60	80.2	6.9

Table 5. Comparison of frequencies of alleles at MDH and PEP enzyme loci in isolates of *H. influenzae* (ETs 1–80) in two populations of neonates: 16 neonates heavily colonized and 13 neonates with invasive disease (meningitis and/or septicæmia)

There was no significant difference for the eight other enzymes tested.

Locus and alleles	Neonates heavily colonized	Neonates with invasive disease	χ^2 test
MDH: 1	0.125	0.077	$\chi^2 = 13.02$ $P < 0.02$
2	0.187	0.077	
3	0.000	0.077	
4	0.187	0.770	
5	0.500	0.000	
PEP: 1	0.000	0.384	$\chi^2 = 10.62$ $P < 0.05$
2	0.375	0.384	
3	0.062	0.000	
4	0.500	0.154	
5	0.062	0.077	

from Canada was recovered from the CSF of a neonate.

Comparison of the allele frequencies of the nine strains recovered from mother–infant invasive infections to those of strains isolated from heavily colonized children revealed no significant differences.

Discussion

Our analysis demonstrated that a large variety of chromosomal genotypes of *H. influenzae* may cause urogenital, maternal and neonatal disease. Indeed, genetic diversity at enzyme loci in the present sample of

isolates is fully equivalent to that recorded in the highly polymorphic species *Escherichia coli* and *Neisseria meningitidis* (Musser *et al.*, 1986*b*).

The number of isolates examined in this study, and belonging to ETs 1 to 80 (Fig. 1) representing *H. influenzae* (*sensu stricto*), was adequate to permit a conclusion that for many of the non-invasive genital infections (e.g. endometritis, urethritis, Bartholinitis and cervicitis) there was little, if any, obvious association with the genetic structure of the pathogen. For example, isolates from cervicitis and endometritis were distributed throughout most of the dendrogram, with no conspicuous pattern. However, for ETs 1 to 80, 69% of strains

responsible for invasive neonatal diseases were clustered in only two phylogenetic lineages characterized by a high frequency of occurrence of the 4 allele of MDH. As demonstrated for *E. coli*, certain alleles of metabolic enzymes may serve as molecular markers for clusters of unusually pathogenic isolates (Goullet & Picard, 1986). Our results indicate that neonatal septicaemia and/or meningitis episodes are frequently due to strains of serologically non-typable *H. influenzae* assigned to two relatively unrelated clusters of allied genotypes.

Strains of the very divergent cluster B (ETs 81 to 85) were strongly associated with disease syndromes that are most unusual for *Haemophilus*: most isolates of these ETs were recovered from neonates or mothers with bacteraemia or from the gastric aspirates of neonates at risk of invasive infections. Lack of invasive disease in Tours caused by strains assigned to this group could perhaps be due to differences in clinical management between the USA and France, since strains from both countries were phenotypically (Quentin *et al.*, 1989) and genotypically relatively homogeneous.

Multilocus enzyme electrophoresis estimates overall genetic relatedness among bacterial isolates from examination of allelic variation at a small fraction of the structural gene loci of the chromosomal genome. For strains of *Legionella* (Selander *et al.*, 1985), *E. coli* and *Shigella* spp. (Ochman *et al.*, 1983), *Bordetella* spp. (Musser *et al.*, 1986b), *Neisseria* (Chun *et al.*, 1985), and the oral streptococci (Gilmour *et al.*, 1987), estimates of genetic relatedness based on multilocus enzyme electrophoresis have been shown to be strongly correlated ($r = 0.8-0.9$) with measures of similarity in total nucleotide sequences derived from DNA hybridization experiments. In general, for samples of 12 to 30 enzymes, strains diverging from one another at a genetic distance greater than 0.55 show a degree of nucleotide sequence similarity well below the 70% level now conventionally taken as the criterion of species limits in many groups of bacteria (Brenner, 1984; Wayne *et al.*, 1987). ETs 81 to 85 diverge from other strains of *H. influenzae* at a genetic distance of 0.84 and the results from DNA hybridization experiments undoubtedly demonstrate that isolates assigned to these ETs represent a previously unrecognized *Haemophilus* species. The failure to identify ETs 81 to 85 among more than 2500 encapsulated isolates of *H. influenzae* (Musser *et al.*, 1988a, b), 60 serologically nontypable isolates recovered from otitis and invasive diseases in children older than one month (Musser *et al.*, 1986a), and more than 200 strains isolated from the respiratory tract and other infections (Mendelman *et al.*, 1987; J. M. Musser, unpublished) strongly suggests that strains of this new species possess specific tropism for the urogenital tract.

For a variety of bacterial species, phenotypic or

natural history correlates of genetic structure have been recorded (Selander & Musser, 1990; Musser *et al.*, 1987). The data presented here confirm earlier observations (Musser *et al.*, 1985, 1986a) that, for *H. influenzae*, little information bearing on the genetic diversity and relationships of isolates is provided by biotyping. Epidemiological or other studies (e.g. antimicrobial testing: Quentin *et al.*, 1988) employing biotyping as the sole criterion for grouping strains must be performed with the knowledge that strains classified in this manner are genetically heterogeneous. Strains of biotype IV belonging to the cryptic species of *Haemophilus* (ETs 81 to 85) constitute a notable exception. They are genetically relatively homogeneous, have identical outer-membrane protein profiles which differ from those of *H. influenzae* and *H. parainfluenzae*, possess peritrichous fimbriation, and are, on average, more susceptible to quinolones than are isolates of *H. influenzae* (Quentin *et al.*, 1988, 1989). Additional characterization will be necessary to complete the description of this newly identified species and will most probably reveal other additional genotypic and phenotypic differences when compared to populations of *H. influenzae* (*sensu stricto*).

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