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Characterization of *Streptococcus agalactiae* Strains by Multilocus Enzyme Genotype and Serotype: Identification of Multiple Virulent Clone Families That Cause Invasive Neonatal Disease

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The chromosomal genotypes of 277 isolates of 16 serotypes of *Streptococcus agalactiae* were characterized by analysis of electrophoretically demonstrable allele profiles at 12 metabolic enzyme loci. The collection comprised the type strain and 276 strains recovered from French symptomatic and asymptomatic subjects. Sixty-one distinctive electrophoretic types (ETs), representing multilocus clonal genotypes, were identified. Cluster analysis of the ETs revealed two primary phylogenetic divisions separated by a genetic distance of 0.62. Division I contained 67 isolates which could be assigned to 13 ETs. Twenty-seven of these isolates were from samples of cerebrospinal fluid (CSF) from neonatal meningitis patients. Two ETs, separated by a genetic distance of 0.217, contained 26 of these 27 isolates. Division II contained 210 isolates, of which 27 were isolated from CSF. This division was more polymorphic and included 48 ETs. Spanning a genetic distance of 0.3, three clusters and one ET were identified within this group. Twenty-four of 27 strains isolated from CSF belonged to one cluster, and 19 of them belonged to two adjacent ETs with a genetic distance of 0.083. Fifty-five of the 68 serotype Ia strains and 24 of the 26 serotype Ib strains were each confined to one of the evolutionary lineages, and 85 of the 86 strains which carried protein antigen c belonged to phylogenetic division II. Most of the type III organisms were assigned to two clone families. The characteristics of this French population argue for the existence of particular groups of strains responsible for neonatal meningitis and demonstrate that serotyping can supply information about the genetic distribution of strains.

Streptococcus agalactiae originating from maternal vaginal carriage is the most common cause of invasive disease in neonates (1, 3, 7, 8, 20). Each year in the United States, *S. agalactiae* infections during pregnancy affect about 50,000 women (6, 7), and invasive infections in neonates account for approximately 11,000 cases of meningitis, with a mortality rate of 15 to 20% (1, 2). In our hospital in France, *S. agalactiae* is responsible for half the neonatal infections (4, 5).

Group B streptococci are differentiated from other beta-hemolytic streptococci by Lancefield serological typing (16). *S. agalactiae* subgroups are determined by serotyping on the basis of cell wall polysaccharides. Thus, isolates are subclassified into serotypes Ia, Ib, II, III, IV, and V and two candidate serotypes, NT6 and 7271 (14, 17, 18, 21, 24). These serotypes may be further classified according to the presence of protein antigen c, R, or X (9, 23).

Epidemiological studies in the United States have shown that more than two-thirds of all cases of *S. agalactiae*-related neonatal diseases are caused by strains producing type III antigen (3). Therefore, serotyping was proposed as a means of predicting the risk of invasive disease. This approach had two pitfalls: (i) serotype III is very frequent in asymptomatic carriage and (ii) in some countries, serotypes other than type III are frequently isolated in early-onset disease (10). An investigation of the genetic diversity of *S. agalactiae* species would therefore be valuable to determine whether particular genotypes correlate with particular diseases.

In the United States, it has been demonstrated that strains synthesizing type III polysaccharide belong to two distantly related evolutionary lineages that apparently differ in their ability to invade the central nervous system. A single clone with an unusually high degree of virulence was responsible for a large proportion of the morbidity and mortality caused by type III *S. agalactiae* (22). A recent study of Danish clinical isolates confirmed the clonal structure of *S. agalactiae* populations but differed noticeably on two points: (i) the population included a major phylogenetic group of serotype III strains that was not represented in the U.S. collection and (ii) strains isolated from the cerebrospinal fluid (CSF) of infants with meningitis did not cluster in particular groups (11). Therefore, the relationship between infection and the genetic structure of the *S. agalactiae* population remains unclear.

Investigation of the chromosomal genetic diversity of national collections of strains could help us understand the genetic structure and complexity of *S. agalactiae*. We gathered a large collection of 276 unrelated *S. agalactiae* strains isolated from cases of various pathologies including a national collection of 54 strains isolated from the CSF of neonates. These strains were studied by multilocus enzyme electrophoresis with the objectives of detecting possible associations of distinctive bacterial genotypes with systemic infections and measuring genetic variation in relation to serotype.

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MATERIALS AND METHODS

Bacterial isolates. The *S. agalactiae* collection was composed of 276 strains obtained between 1986 and 1990 in France (10, 12). Isolates were from various sources: 111 strains were isolated from the genital tracts of asymptomatic women, 54 strains were from the CSF of newborn children with meningitis (45 strains from early-onset disease and 9 from late-onset disease), 10 were from endocervical tissues, 5 were from blood cultures, 14 were from infections associated with intrauterine devices (IUDs), and 82 were from the gastric fluid of newborn children. The type strain (NCTC 8181) was used as a reference.

The national collection of CSF strains was gathered from the following 25 general hospitals throughout France: Aix (two strains), Angoulême (two strains), Annecy (one strain), Arras (five strains), Aulnay/Bois (two strains), Bondy (two strains), Dole (one strains), Créteil (three strains), Isle of Martinique (three strains), Le Havre (one strain), Le Mans (three strains), Lonjumeau (one strain), Mantes la Jolie (one strain), Moulins (one strain), Mulhouse (one strain), Orléans (one strain), Paris (two strains), Pau (one strain), Poitiers (four strains), Pontoise (two strains), St. Brieuc (eight strains), St. Germain en Laye (two strains), Tours (three strains), Vienne (one strain), and Villeuneuve St. Georges (one strain).

The strains were stored at −80°C in Schaedler-vitamin K_3 broth (bioMérieux, Marcy l'Etoile, France) containing 10% glycerol.

Serotyping. The following 10 immunizing *S. agalactiae* strains of the indicated groups were used: type Ia, 090R; type Ib, H36B; type Ia/c, A909; type II, 18RS21; type III, D136C; type IV, 3139 (originally Rabinowitz); type V, NT1 1169 (originally Wilkinson); protein antigen R, R Compton; protein antigen X, X Compton; and group B, 090R variant. The preparation of vaccines with reference strains, the immunization schedule, control antibodies, and absorption of cross-reacting antibodies were all as previously described (10, 13, 19).

Strains were typed by double diffusion and/or polystyrene latex agglutination (10, 15, 28).

Enzyme electrophoretic analysis. Each isolate was grown overnight at 37°C in 200 ml of brain heart infusion broth (Difco). Cells were harvested by centrifugation at 6,000 × *g* for 10 min at 4°C and resuspended in 1.5 ml of 50 mM Tris–5 mM EDTA (pH 7.5). The bacteria were then sonicated (Sonifier Cell Disruptor, model 200, with microtip; Branson Sonic Power Co., Danbury, Conn.) for 30 s at a 50% pulse with ice-bath cooling. The samples were centrifuged at 20,000 × *g* for 20 min at 4°C, and the supernatant (lysate) was collected and stored at −70°C.

Horizontal starch-gel electrophoresis and the demonstration of specific enzyme activity were performed as described by Selander et al. (26). The following 12 enzymes were assayed: phosphoglucose isomerase, hexokinase, glyceraldehyde 3-phosphate dehydrogenase, carbamate kinase, adenylate kinase, two peptidases (phenylalanylleucine peptidase and leucylglycylglycine peptidase), nucleoside phosphorylase, esterase, phosphoglucomutase 1, phosphoglucomutase 2, and glutamate dehydrogenase.

For each enzyme, distinctive mobility variants (electromorphs) were numbered in order of decreasing rate of anodal migration. Electromorphs of an enzyme were equated with alleles at the corresponding structural gene loci. Because almost all isolates showed activity for all enzymes, the corresponding gene loci are presumed to be located on the chromosome rather than on plasmids. The occasional absence of activity for an enzyme was attributed to the presence of a null allele, designated zero.

Each isolate was characterized by its combination of alleles at the 12 enzyme loci: overall allele profiles, corresponding to unique multilocus genotypes, were designated electrophoretic types (ETs) (26). (The numerical designations of alleles and ETs in this study do not correspond exactly to those used by Musser et al. [22] because there were additional electromorphs in our population.)

Genetic diversity at an enzyme locus (h) among either ETs or isolates was calculated from the allele frequencies as $h = (1 - \Sigma x_i^2)[n/(n-1)]$, where x_i is the frequency of the ith allele and n is the number of ETs (26). The mean genetic diversity per locus (H) is the arithmetic average of h values for all loci. The genetic distances between pairs of ETs were calculated from 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 (26).

RESULTS

A total of 272 clinical *S. agalactiae* isolates were serologically typeable, and 4 isolates were nontypeable. The serotype distribution, classified by source of isolation, is shown in Table 1. The type strain is of serotype II.

Genetic diversity of isolates. For the sample of 277 strains tested, 11 of the 12 enzymes assayed were polymorphic for two to six electrophoretically demonstrable variants; one locus (phosphoglucose isomerase) was monomorphic (Table 2).

A total of 61 distinctive multilocus combinations or ETs were identified. The mean genetic diversity per locus (H) was 0.404 (Table 2).

Thirty-one of the 61 ETs contained more than one isolate (range, 2 to 43). Fifty-seven percent of the strains (158 isolates) clustered in only six ETs: 35 strains in ET 11, 43 strains in ET 12, 18 strains in ET 19, 17 strains in ET 34, 13 strains in ET 49, and 32 strains in ET 58.

Genetic relationships among the 61 ETs are represented in the dendrogram shown in Fig. 1. The smallest observed genetic distance (0.083) corresponds to a single-locus difference. The

TABLE 1. Serotypes of strains of *S. agalactiae*, classified by source of isolation

Serotype	No. of Isolates						Total
	Vagina[a]	Endo cervix[b]	Gastric fluid[c]	IUD[d]	Blood[e]	CSF[f]	
Ia	14	1	9	5	1		30
Ia/c	23	2	6	1	1	5	38
Ib	3	1	8				12
Ib/c	7		4	1		2	14
II	11	1	8	2		2	24
II/c	5		5	2			12
II/R	6		4			2	12
III	12	1	18	1	3	9	44
III/c	1	2					3
III/R	18	1	8			32	59
IV/c	1		1			2	4
V			1	1			2
V/c			1				1
V/R	1						1
NT	1	1	2				4
NT/c	5		7	1			13
NT/R	3						3
Total	111	10	82	14	5	54	276

[a] Vaginas of asymptomatic women.
[b] Endocervices of pregnant women with premature rupture of membranes and/or risk of premature birth.
[c] Neonatal gastric fluid of asymptomatic neonates.
[d] IUDs of women with endometritis.
[e] Blood cultures of neonates with negative cultures of CSF.
[f] CSF of neonates with meningitis.

TABLE 2. Allele frequencies at 12 enzyme loci and genetic diversity among 61 ETs of *S. agalactiae*

Locus (enzyme)[a]	Frequency of indicated allele						h^b
	0	1	2	3	4	5	
PM1		0.754	0.229	0.016			0.386
PM2	0.049	0.049	0.229	0.360	0.295	0.016	0.738
G3P		0.016	0.147	0.688	0.016		0.496
PGI		1.000					0.000
NSP		0.016	0.934	0.049			0.111
CAK		0.574	0.426				0.498
ADK		0.016	0.786	0.196			0.334
GLD	0.016	0.754	0.229				0.386
HEX		0.967	0.032				0.065
PLP		0.606	0.393				0.479
LGG		0.213	0.426	0.344	0.016		0.666
EST	0.049	0.049	0.213	0.442	0.229	0.016	0.697

[a] PM1, phosphoglucomutase 1; PM2, phosphoglucomutase 2; G3P, 3-phosphate dehydrogenase; PGI, phosphoglucose isomerase; NSP, nucleoside phosphorylase; CAK, carbamate kinase; ADK, adenylate kinase; GLD, glutamate, dehydrogenase; HEX, hexokinase; PLP, phenylalanylleucine peptidase; LGG, leucylglycylglycine peptidase; EST, esterase.
[b] $H = 0.404$.

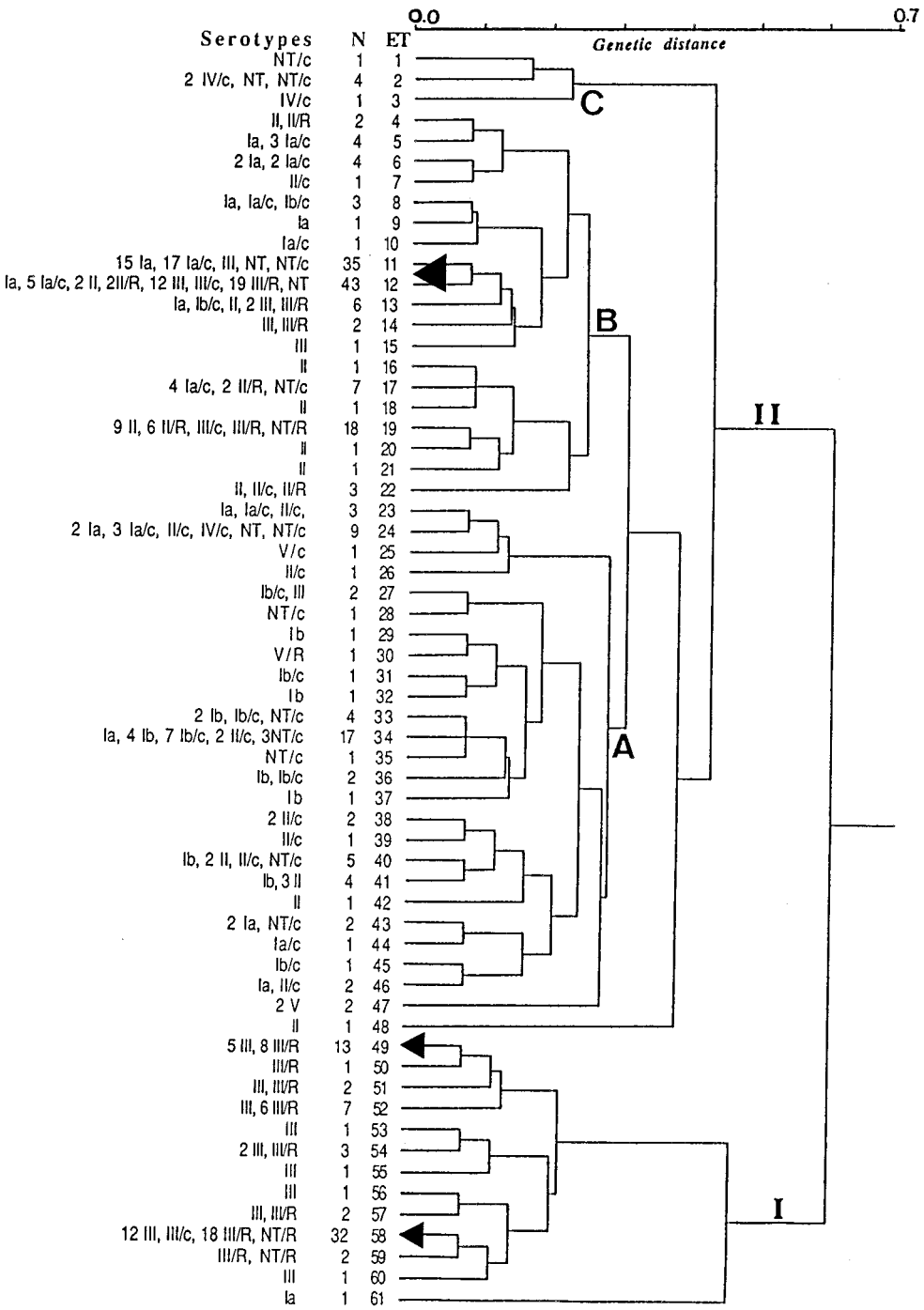

FIG. 1. Genetic relationships among 61 ETs of *S. agalactiae*. The dendrogram was generated by the average-linkage method of clustering from a matrix of coefficients of pairwise genetic distances (26) based on 12 enzyme loci. There are two primary divisions (I and II), and there are four major lineages (A, B, and C and ET 48, containing only the type strain) in division II. N, number of isolates examined. Of the 54 CSF isolates, 45 were assigned to three clone families (◀): ETs 11 and 12 (19 strains), ET 49 (9 strains), and ET 58 (17 strains).

largest distance (0.62) corresponds to differences at 7 of the 12 loci assayed.

Two primary phylogenetic divisions were identified, at a genetic distance of 0.62. Division I contained 67 isolates, which were assigned to 13 ETs. Division II contained 210 isolates and included 48 ETs. Within the latter division, and spanning a genetic distance of 0.3, three clusters and one ET were identified: cluster A contained 68 strains, cluster B contained 135 strains, and cluster C included only 6 strains, and ET 48 was represented by a single strain (the type strain).

Genetic variation in relation to serotype. The serotype distribution for each ET is indicated in Fig. 1; genetic diversities among the isolates of each serotype are shown in Table 3. Strains of serotype III showed the greatest diversity ($H = 0.362$ and 0.385 for serotypes III/R and III, respectively), and strains of serotype II/R were the least variable ($H = 0.104$).

TABLE 3. Mean genetic diversity per locus for 12 enzyme loci among *S. agalactiae* isolates classified by serotype

Serotype	No. of isolates	No. of ETs	Mean no. of alleles	*H*
Ia	30	13	2.0	0.150
Ia/c	38	10	1.6	0.132
Ib	12	8	1.5	0.115
Ib/c	14	8	1.7	0.145
II	25[a]	13	1.8	0.212
II/c	12	10	1.8	0.234
II/R	12	5	1.5	0.104
III	44	16	2.2	0.385
III/c	3	3		
III/R	59	12	1.8	0.362
IV/c	4	3		
V	2	1		
V/c	1	1		
V/R	1	1		
NT	4	4		
NT/c	13	11	1.9	0.261
NT/R	3	3		

[a] Twenty-four clinical isolates and the type strain.

TABLE 4. Mean genetic diversity per locus for 12 enzyme loci among *S. agalactiae* isolates within primary phylogenetic divisions I and II, classified by source of isolation

Division and source of isolation[a]	No. of isolates	No. of ETs	Mean no. of alleles	*H*
I				
Vagina	20	9	1.75	0.159
Endocervix	3			
Gastric fluid	13	6	1.33	0.086
IUD	1			
Blood culture	3			
CSF	27	3	1.16	0.046
II				
Vagina	91	29	2.2	0.220
Endocervix	7			
Gastric fluid	69	31	2.5	0.235
IUD	13	6	1.8	0.201
Blood culture	2			
CSF	27	8	1.8	0.131

[a] Gastric fluid and CSF samples were from neonates.

All serotypes were represented by strains of a variety of multilocus enzyme genotypes, with the exceptions of serotypes V, V/c, and V/R, for which the sample sizes were small. The number of ETs per serotype ranged from 5 for type II/R to 16 for type III, and the mean number of alleles per locus among isolates of a serotype varied from 1.5 for types Ib and II/R to 2.2 for type III.

Sixty-four of the 106 strains of serotype III (types III, III/c, and III/R) belonged to primary division I, and 42 strains belonged to primary division II. In phylogenetic division II, 32 of the 42 strains of serotype III represented ET 12. All strains of other serotypes belonged to primary division II, with the exception of one strain of serotype Ia. Twenty-four of the 26 strains of serotype Ib (Ib and Ib/c) were in cluster A, and 55 of the 68 strains of serotype Ia (Ia and Ia/c) were in cluster B. Serotype II was found in the two major lineages, A and B.

The expression of protein antigen R did not correlate with the ET or group. In contrast, 85 of the 86 strains that expressed protein c belonged to phylogenetic division II.

Genetic variation of strains in relation to clinical episodes. Strains isolated from vaginal tissue, endocervical tissues, gastric fluid, and IUD-associated infections did not cluster into particular phylogenetic groups.

Fifty-four strains were isolated from neonates during episodes of meningitis (CSF; Table 1). Half of these invasive strains were in primary phylogenetic division I, and the other half were in primary phylogenetic division II.

In contrast to strains isolated from other anatomic sites, CSF isolates were assigned to a small number of clusters in primary phylogenetic divisions I and II. In the first division, most of the CSF isolates represented ET 49 (1 type III strain and 8 type III/R strains) or ET 58 (2 type III strains and 15 type III/R strains). These two ETs were separated by a genetic distance of 0.217. In the second division, strains belonging to major lineage A were rarely involved in invasive disease (only 1 [ET 34] of 67 strains). In this division, 24 of the 27 strains responsible for meningitis were in lineage B (Fig. 1), and 19 of those strains clustered in two closely related ETs (ETs 11 and 12) at a genetic distance of 0.083. CSF isolates of ET 11 were of type Ia/c (four strains) or type III (one strain), and CSF isolates of ET 12 were of type Ia/c (one strain), type III (four strains), or type III/R (nine strains). Other CSF isolates belonged to various ETs of division II: ET 2 (one type IV/c strain), ET 3 (one type IV/c strain), ET 13 (one type Ib/c strain), ET 17 (two type II/R strains), ET 19 (two type II strains), and ET 34 (one type Ib/c).

The distribution among the ETs of the nine CSF strains isolated from late-onset disease (two strains in ET 12, four strains in ET 58, and one strain each in ETs 13, 34, and 49) was not significantly different from that of CSF strains isolated from early-onset disease ($\chi^2 = 12.96$; $P = 0.23$).

No correlation between geographic origin and clustering of CSF strains was observed. For example, from St. Brieuc hospital, where the largest number of CSF strains was obtained, the eight strains isolated from CSF were in ET 12 (two strains), ET49 (one strain), ET 58 (two strains), ET 19 (one strain), ET 34 (one strain), and ET 52 (one strain).

The mean genetic diversity per locus was calculated for the CSF strains within each phylogenetic division (Table 4). The CSF isolates in each phylogenetic group were closely related ($H = 0.046$ and 0.131 in primary phylogenetic divisions I and II, respectively).

DISCUSSION

S. agalactiae is an important human pathogen. Examination of allelic variation at enzyme loci provides a basis for estimating overall levels of single-locus and multilocus genotypic variation in a species. It is a valuable first step towards correlating specific genotypes with particular diseases (27) and towards ascertaining the degree to which cell wall polysaccharides and protein types reflect the genetic structure of populations. Because serological methods have been extensively employed by clinical microbiologists, this genetic validation of serotyping was of importance.

Our analysis of 277 strains at 12 enzyme loci demonstrated that a variety of chromosomal genotypes may cause urogenital, maternal, and neonatal diseases. However, the genetic diversity of *S. agalactiae* strains appeared to be less pronounced than that described for other polymorphic species responsible for neonatal infections, such as *Escherichia coli* and *Haemophilus influenzae* (25, 27). Only 61 ETs were identified in a sample of 277 strains, and 57% of the strains clustered in only 6 ETs.

Electrophoretically demonstrable allelic variation of 11 enzyme loci in 128 isolates of *S. agalactiae* recovered predominantly from humans in the United States has previously been

described (22). Clearly, both in France and in the United States, the population of *S. agalactiae* strains isolated from humans can be divided into two phylogenetic divisions (I and II), with three major lineages (A, B, and C) being represented in phylogenetic division II. Primary division I appears to be more polymorphic in France, with three groups of ETs at a genetic distance of 0.2 and an additional ET at a genetic distance of 0.466. If these features were characteristic of the population in the United States, they would have been detected by the analysis of 11 enzymes.

Serotyping based on cell wall polysaccharides and protein antigens provided some information on the genetic diversity and relationships of isolates. Specifically, 92% of isolates of serotype Ib (Ib and Ib/c) were confined to ETs within only one of the evolutionary lineages of division II (cluster A), and 81% of the isolates of serotype Ia (Ia and Ia/c) were distributed in the other major evolutionary lineage of division II (cluster B). In addition, the presence of protein antigen c appears to be a marker of phylogenetic division II.

For many of the noninvasive genital infections, there was little, if any, obvious association with the genetic structure of the pathogen population. For example, isolates from cases of endometritis associated with IUDs were distributed throughout most of the dendrogram, with no conspicuous pattern.

Our data demonstrated that neonatal meningitis episodes were frequently caused by strains of a small number of clone families, whereas strains isolated from the vaginas of asymptomatic pregnant women and from gastric fluid did not cluster. This finding suggests that strains of *S. agalactiae* originating from the genital tract can invade amniotic fluid during labor but are not all able to infect the central nervous system. Therefore, vaginal colonization should not be systematically considered a risk for neonatal infection, and the prescription of antibiotics for vaginal carriage is probably not useful in many cases.

There are some differences in the relationships of neonatal invasive strains between France and the United States. The French invasive strains were equally distributed between divisions I and II, whereas 81% of invasive strains in the United States belonged to division I. This genetic finding agrees with results of serotyping, which show a comparable difference in the relationships of serotypes in neonatal strains between the two countries: serotype III, which composed division I, was greatly predominant in neonatal disease in the United States (more than two-thirds of strains), whereas this serotype represented only 51% of the strains isolated in early-onset disease in France (3, 10). Moreover, in the United States, division I was represented by a single ET containing isolates mainly from symptomatic neonates. In contrast, in France, division I was more polymorphic (13 ETs) and contained strains of various origins, including two major groups of neonatal invasive strains (ETs 49 and 58). Apparently, more genetic events have occurred in the European population of *S. agalactiae*, leading to the emergence of two virulent clones in the population belonging to this phylogenetic division. National differences in use of antibiotics during pregnancy and delivery, ethnic distribution, type of neonate feeding, and levels of maternal antibodies against *S. agalactiae* may all contribute to the difference in bacterial evolution.

In conclusion, our results argue for the existence of particular groups of *S. agalactiae* strains that are responsible for neonatal meningitis in France and demonstrate that serotyping provides information about the overall genetic character of strains. Further research is needed to determine convenient phenotypic, metabolic, or genotypic criteria for identifying virulent clone families among strains present in the genital tract. This would be valuable for obstetricians and could help them make early and accurate preventive decisions during deliveries with a high risk of infection.

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