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Population Structure of Invasive and Colonizing Strains of *Streptococcus agalactiae* from Neonates of Six U.S. Academic Centers from 1995 to 1999[▽]

John F. Bohnsack,[1]* April Whiting,[1] Marcelo Gottschalk,[2] Diane Marie Dunn,[3] Robert Weiss,[3] Parvin H. Azimi,[4] Joseph B. Philips III,[5] Leonard E. Weisman,[6] George G. Rhoads,[7] and Feng-Ying C. Lin[8]

Department of Pediatrics[1] and Genome Center,[3] University of Utah Health Sciences Center, Salt Lake City, Utah; Faculté de Médicine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec, Canada[2]; Department of Infectious Diseases, Children's Hospital Medical Center of Northern California, Oakland, California[4]; University of Alabama at Birmingham, Birmingham, Alabama[5]; Baylor College of Medicine, Houston, Texas[6]; University of Dentistry and Medicine of New Jersey, Piscataway, New Jersey[7]; and National Institute of Child Health and Human Development, National Institutes of Health, Department of Health and Human Services, Bethesda, Maryland[8]

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The purpose of this study was to describe the population structure of group B streptococci (GBS) isolated from infected and colonized neonates during a prospective active-surveillance study of early-onset disease in six centers in the United States from July 1995 to June 1999 and to examine its relationship to bovine strains of GBS. The phylogenetic lineage of each GBS isolate was determined by multilocus sequence typing, and isolates were clustered into clonal complexes (CCs) using the eBURST software program. A total of 899 neonatal GBS isolates were studied, of which 129 were associated with invasive disease. Serotype Ia, Ib, and V isolates were highly clonal, with 92% to 96% of serotype Ia, Ib, and V isolates being confined to single clonal clusters. In contrast, serotype II and III isolates were each comprised of two major clones, with 39% of serotype II and 41% of serotype III isolates in CC 17 and 41% of serotype II and 54% of serotype III isolates in CC 19. Further analysis demonstrates that the CC 17 serotype II and III GBS are closely related to a previously described "ancestral" lineage of bovine GBS. While 120 (93%) of invasive GBS were confined to the same lineages that colonized neonates, 9 (7%) of the invasive GBS isolates were from rare lineages that comprised only 2.7% of colonizing lineages. These results are consistent with those for other geographic regions that demonstrate the highly clonal nature of GBS infecting and colonizing human neonates.

Streptococcus agalactiae (group B streptococci [GBS]), a primary cause of bovine mastitis, emerged in the 1960s as a significant human pathogen that causes neonatal sepsis and meningitis (1). Despite a recent decline in incidence, GBS remains a leading cause of neonatal mortality and morbidity in the United States (2, 7, 29, 30). GBS colonizes the genitourinary and gastrointestinal tracts of about a third of healthy adult women (24). Neonatal disease results from transmission of the organism from the pregnant mother to the neonate. Heavy colonization of the neonate and maternal chorioamnionitis are known risk factors for neonatal infection (10, 20).

GBS can be divided into nine serotypes based on the immunologic reactivity of the capsular polysaccharide. The structure of GBS capsular polysaccharide is determined by genes in the *cps* locus which encode enzymes responsible for the synthesis of the polysaccharides (8, 34, 35). Investigators using a variety of techniques have demonstrated that the population of GBS that colonizes and infects humans is comprised of a relatively small number of lineages (4, 12–16, 23, 26, 28, 31, 32). Each serotype is usually distributed among several different lineages, and each lineage may contain several different serotypes, indicating that serotype alone is not a sufficient marker to identify a phylogenetic lineage and suggesting that serotype switching occurs within lineages, presumably by horizontal transfer of *cps* genes (4, 16, 23).

Multilocus sequence typing (MLST) has become the standard method for determining the population structure of GBS and has been applied to the molecular epidemiology of GBS infections by several investigators (4, 9, 15, 16, 22, 23). We recently reported the relationship between the phylogenetic lineages that comprise serotype III GBS isolates and the occurrence of neonatal early-onset disease in a NICHD multicenter prospective study of GBS disease and colonization in the newborns. Our data showed that clonal complex 17 (CC 17) serotype III GBS were associated with early-onset GBS disease after adjusting for cord serum immunoglobulin G (IgG) anti-GBS type III and other potential confounding factors (22). In the current study, we analyzed the phylogenetic lineages of the invasive and colonizing strains of the remaining serotypes from the NICHD multicenter study, and we describe the population structure of invasive and colonizing GBS type Ia, Ib, II, III, and V strains. We also examined the genetic relationship of human strains to bovine strains of GBS.

* Corresponding author. Mailing address: University of Utah Health Sciences Center, Department of Pediatrics, 50 North Medical Dr., Salt Lake City, UT 84132. Phone: (801) 587-7416. Fax: (801) 587-7417. E-mail: john.bohnsack@hsc.utah.edu.

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MATERIALS AND METHODS

Subjects. The newborn population from which the GBS were isolated has been described previously (18–22). Briefly, these isolates are from the NICHD seroepidemiological studies of early-onset disease (EOD) caused by GBS conducted between July 1995 and June 1999 in six U.S. academic centers: The University of Alabama at Birmingham, Birmingham, AL; Baylor College of Medicine, Houston, TX; Columbia University, New York, NY; University of Florida, Gainesville, FL; University of Medicine and Dentistry of New Jersey, New Brunswick, NJ; and Children's Hospital Medical Center of Northern California, Oakland. Infants with EOD, diagnosed by isolation of GBS from the blood or cerebrospinal fluid within 7 days of birth, were identified by prospective active surveillance of neonatal intensive care nurseries and microbiology laboratories. A total of 132 neonates with EOD were identified among 138,740 live births (0.95/1,000 live births). Colonized infants were identified by obtaining swabs for culture from the throat, anus, umbilicus, and external ear canals at birth, before the first bath, from a sample of newborns each month. Samples from a total of 17,690 neonates were cultured, and 1,674 (9.5%) of these neonates were colonized but did not develop EOD (19).

Bacterial isolates. Bacterial isolates were from infants with EOD caused by GBS (cases) or from infants born within 6 months of the case at the same study center who were colonized at birth by the same serotype of GBS as the cases but did not develop GBS disease (controls). Three of the invasive GBS isolates could not be typed by immunological methods and were not included in this study. For each case, up to four heavily colonized (GBS positive in at least three of four sites) and four lightly colonized (GBS positive in one to two sites) infants were selected. One strain from each infant was included in the analysis. In total, 129 isolates from infants with early-onset GBS disease, 447 isolates from lightly colonized infants, and 323 isolates from heavily colonized infants were included in this study. Also included in this study were restriction digest pattern (RDP) type II-1, II-2, and II-3 GBS from a separate sample of previously described human isolates (32) and a sample of bovine serotype II (18 isolates) and III (38 isolates) GBS from a large collection of GBS isolated from bovine milk in Quebec Province, Canada, during 1996 and 1997 (25). Bacterial isolates, frozen in Todd-Hewitt broth, were sent to the University of Utah, where the isolates were thawed and an inoculum streaked on blood-agar plates. A single colony was picked and grown overnight in Todd-Hewitt broth. DNA was isolated from the overnight culture using the Qiagen DNeasy tissue kit (Qiagen, Valencia, CA) and stored at 4°C until use.

MLST and assignment to clonal clusters. Multilocus sequence typing (MLST) was carried out as described previously (15). Briefly, PCR was used to amplify small (~500-bp) fragments from seven housekeeping genes (*adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK*, and *tkt*) chosen on the basis of their chromosomal location and sequence diversity. The seven PCR products were purified and sequenced and an allele number assigned to each fragment based on its sequence. Each isolate was assigned a sequence type (ST) based on the allelic profile of the seven amplicons. The sequences of all novel alleles and the composition of novel STs identified in this study are available at http://sagalactiae.mlst.net. An unrooted dendrogram showing the relationship among STs was constructed using the MEGA software program, version 3.1 (17).

Strains were grouped into CCs using the eBURST software program (11). The term "singleton ST" refers to an ST that did not cluster into a CC.

The central portion of *infB* was amplified from bacterial DNA by PCR with oligonucleotide primers as described by Hedegaard et al. (13). Each amplicon was purified and sequenced.

Detection of mobile genetic elements. The mobile genetic elements IS*1548*, GBSi1, and IS*1563* were detected by PCR using sequence-specific primers for flanking regions of the AW-10, *scpB-lmb*, and *hylB* insertion sites and agarose gel electrophoresis to determine the sizes of the PCR products, as described previously (6, 32).

RESULTS

STs and CCs of human neonatal strains. MLST was performed on 899 GBS isolates from the neonates (129 invasive and 770 colonizing GBS). A total of 78 individual STs were identified (Fig. 1). Sixty-four of the STs were clustered in 7 CCs, while 14 were singleton STs (Table 1; Fig. 2). Ninety-five percent (856/899) of the isolates were found within 5 of the 7 CCs: CC 1, CC 12, CC 17, CC 19, and CC 23. Greater than 90% of GBS in CC 1 and CC 23 had a single ST (ST 1 and ST

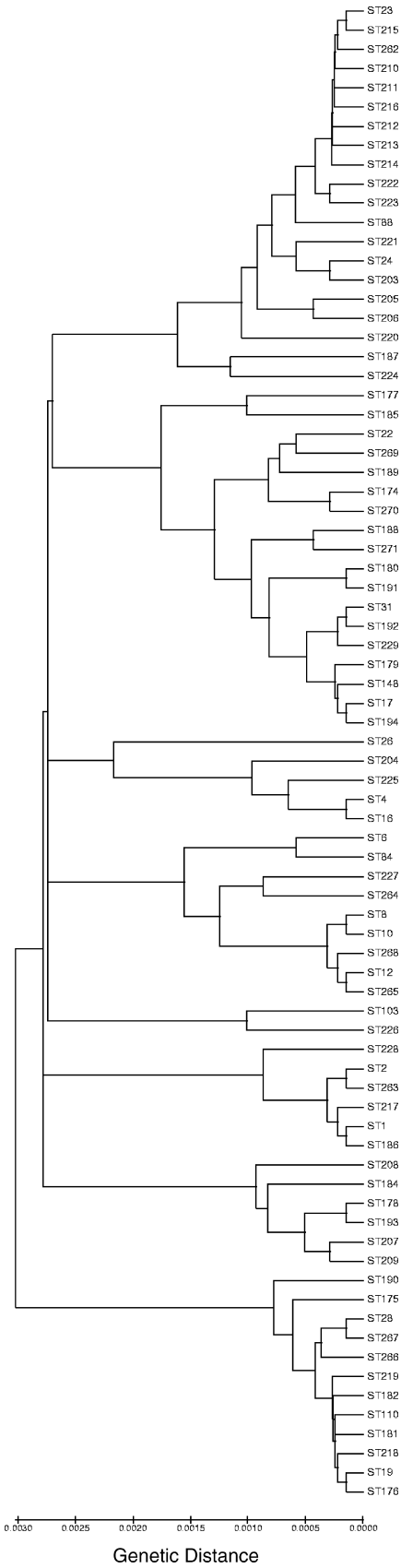

FIG. 1. Dendrogram of the STs of the human GBS isolates characterized in this study.

TABLE 1. STs and serotypes comprising CCs of invasive and colonizing GBS from neonates of six U.S. academic centers

CC (n^a)	ST (n^b)	No. (%^{c}) of isolates	No. of isolates with serotype				
			Ia	Ib	II	III	V
1 (147)	1	137 (93)		2		1	134
	Other (5)	10 (7)			3	1	6
4 (4)	4	3 (75)	3				
	16	1 (25)	1				
12 (85)	8	33 (39)		33			
	12	47 (55)		41	4	2	
	Other (4)	5 (6)		4	1		
17 (114)	17	60 (53)				60	
	22	20 (18)			20		
	31	10 (9)				10	
	180	10 (9)				10	
	Other (12)	14 (12)			2	12	
19 (151)	19	109 (72)			4	103	2
	28	18 (12)			18		
	Other (11)	24 (16)			1	20	3
23 (359)	23	329 (92)	325			2	2
	Other (16)	30 (8)	28		1	1	
207 (5)	184	1 (25)				1	
	207	2 (25)					2
	208	1 (25)					1
	209	1 (25)					1
Singletons (34)[d]	Various (14)	34	26	1	2	3	2
Total			383	81	56	226	153

[a] *n*, no. of isolates.
[b] *n*, no. of STs.
[c] Percentage of isolates of that ST in the CC.
[d] Twenty of the singleton strains belonged to ST-24, which sometimes clusters with CC 23 (16).

23, respectively), while the three other CCs were not as dominated by a single ST: 94% of the isolates in CC 12 were in two STs (ST-12 and ST-8), 84% of the isolates in CC 19 were in two STs (ST-19 and ST-28), and 89% of the isolates in CC 17 were in four STs (ST-17, ST-22, ST-31, and ST-180).

Distribution of serotypes among CCs. GBS of each serotype were distributed among multiple CCs or singleton STs, but the majority of serotype Ia, Ib, and V isolates were each confined to single CCs: 92% of serotype Ia GBS were in CC 23, 96% of serotype Ib GBS were in CC 12, and 92% of serotype V GBS were in CC 1 (Table 1). In contrast, 82% of serotype II isolates and 95% of serotype III isolates were distributed between two CCs: 39% of serotype II GBS were in CC 17, and 43% of serotype II GBS were in CC 19, while 40% of serotype III GBS were in CC 17 and 54% of serotype III GBS were in CC 19 (Table 1).

Multiple serotypes were found within 9.6% of individual STs, suggesting that serotype switching had occurred in these STs, but a single serotype predominated in every ST that contained multiple serotypes, e.g., serotype V in ST-1, serotype Ib in ST-12, serotype III in ST-19, and serotype Ia in ST-23 (Table 1). Thus, there was a tendency for a single serotype to predominate within individual STs.

Distributions of clones in invasive and colonizing strains of GBS from neonates. A total of 129 isolates were from neonates with invasive disease, and 770 isolates were from neonates who were colonized with GBS at birth but did not develop early-onset GBS disease. As might be expected, 94% of the invasive isolates were found in the five major CCs (CC 1, CC 12, CC 17, CC 19, and CC 23) that comprise 95% of the overall sample (Table 2). Almost all the invasive serotype Ia, Ib, or V strains were from single CCs: 90% of invasive serotype Ia strains were in CC 23, 94% of serotype Ib strains were in CC 12, and 96% of invasive serotype V strains were in CC 1 (Table 2). Three of the invasive serotype Ia strains were in ST-24, which is closely related to the STs in CC 23 and actually clustered in CC 23 in a previous analysis of a different sample of GBS isolates (16). Invasive serotype Ib GBS from CC 12 GBS were equally divided between ST-8 and ST-12 strains at a ratio that was not significantly different from the percentage of colonizing strains that were in ST-8 and ST-12. Hence, the percentage of serotype Ia, Ib, and V GBS clones from neonates with EOD in this sample is similar to the percentage of the clones of the corresponding serotype in the colonized neonates.

We previously showed that serotype III GBS from CC 17 in this sample were associated with the occurrence of invasive disease after adjusting for cord serum IgG anti-GBS serotype III (22). Thirty-nine percent of the serotype II GBS in this study were from CC 17, an unusual finding since serotype II GBS from CC 17 have only rarely been identified among sam-

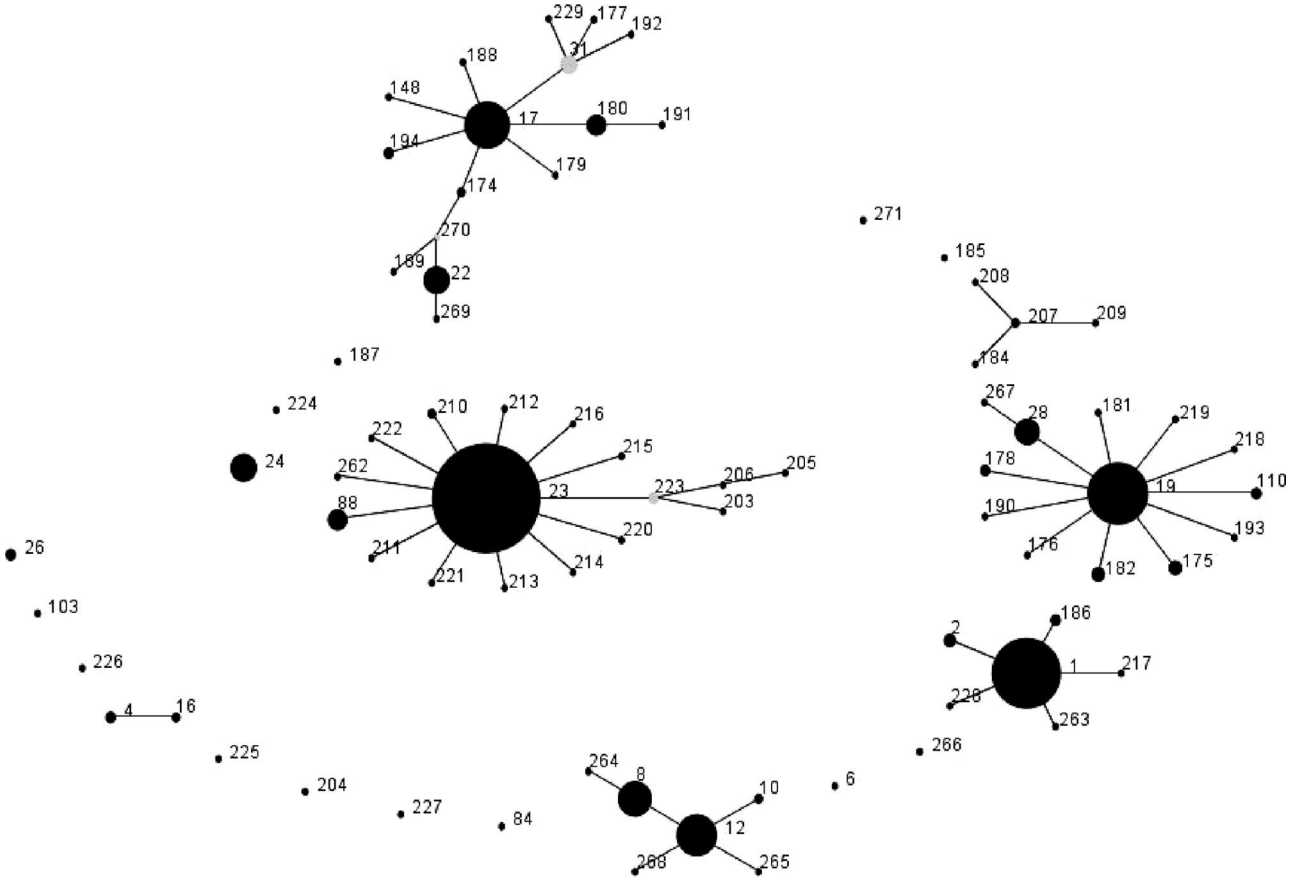

FIG. 2. CCs and singletons of the 899 neonatal GBS isolates in this study. The CCs were determined by using the eBURST software program (see Materials and Methods).

ples from other geographic regions (4, 15, 16, 23). Similar to findings for serotype III GBS, the invasive serotype II GBS in this study were found more frequently to be from CC 17: 67% (6/9 isolates) of invasive type II GBS were in CC 17, compared to only 34% (16/47 isolates) of colonizing type II GBS. The difference was not significant ($P = 0.09$, chi-square test) (Table 2).

Clones rarely associated with EOD also rarely colonize neonates. Although the majority of invasive and colonizing GBS were from five CCs, each serotype also had rare clones, which were defined as isolates from outside the major CCs or as isolates from within the five major CCs but with a serotype different from the predominant serotype in the CC. Seven rare clones were identified among 9 invasive isolates and 21 colonizing isolates and 22 additional rare clones in 38 colonizing isolates. Seven percent of invasive isolates, compared with 2.7% of colonizing strains, were from 7 rare clones ($P = 0.02$, chi-square test) (Table 3). In three cases, the rare clones were

TABLE 2. Distributions of CCs in invasive and colonizing GBS from neonates at six U.S. academic centers[a]

CC	No. of isolates with serotype										Total no. of isolates	
	Ia		Ib		II		III		V			
	Inv	Col	Inv	Col	Inv	Col	Inv	Col	Inv	Col	Inv	Col
1			1	1	1	2		2	22	118	24	123
12			15	63		5		2			15	70
17		1			6	16	7	75			23	92
19					2	21	11	112		5	13	138
23	47	306				1		3		2	47	312
Other or singleton[b]	5	24		1		2	1	2	1	6	7	35
Total	52	331	16	65	9	47	29	196	23	131	129	70

[a] Inv, invasive; Col, colonizing.
[b] Composition of the remaining invasive isolates: three serotype Ia isolates with ST-24, one serotype Ia isolate with ST-16 (singleton), one serotype Ia isolate with ST-204 (singleton), one serotype III isolate with ST-184 (CC 207), and one serotype V isolate with ST-26 (singleton).

TABLE 3. Rare clones identified for invasive and colonizing isolates

Clone	No. (%) of invasive isolates[a]	No. (%) of colonizing isolates[b]
Ia/ST-16	1 (1.9)	3 (0.9)
Ia/ST-24	3 (5.8)	16 (4.8)
Ia/ST-204	1 (1.9)	0 (0)
Ib/ST-1	1 (6.2)	1 (1.5)
II/ST-263	1 (11.1)	0 (0)
III/ST-184	1 (3.4)	0 (0)
V/ST-26	1 (4.3)	1 (0.8)
Other rare clones (n = 22)	0 (0)	38 (4.9[c])

[a] Percentage of invasive isolates of this serotype in this clone.
[b] Percentage of colonizing isolates of this serotype in this clone.
[c] Percentage of isolates carrying one of these 22 rare clones among 770 colonizing isolates.

found in invasive isolates but not in colonizing isolates. In most instances, rare clones made up a higher percentage of the invasive isolates of that serotype than did the colonizing isolate of the serotype. The exception was the ST-24 serotype Ia isolates, which—like the closely related CC 23 serotype Ia strains–were represented at a similar rate among invasive and colonizing serotype Ia strains.

Relationship of CC 17 serotype II GBS to bovine GBS. Previous MLST analysis of human and bovine GBS demonstrated that serotype III GBS from CC 17 are closely related to a lineage of bovine GBS (3). We examined the relationship of human CC 17 serotype II GBS to bovine GBS by analyzing serotype II bovine GBS from a large sample of previously characterized isolates (6, 25). MLST revealed that 15 of the 19 serotype II bovine strains examined were ST-61 or ST-67 (Table 4). The ST distributions of the 38 strains of serotype III bovine GBS are also shown in Table 4. Analysis of the human and bovine GBS by use of eBURST clustered bovine serotype II and III strains from ST-17, ST-61, and ST-67 with the human CC 17 serotype II and III strains into a single clonal cluster (Fig. 3).

CC 17 and CC 19 serotype II and serotype III GBS comprise at least four genetically distinct clones. While serotype II and III GBS share the same CCs, it should be noted that the STs of these two serotypes in both CCs largely do not overlap, suggesting that the serotype II and serotype III GBS within CC 17 and CC 19 from this sample are largely distinct. We previously showed in a different sample that serotype II and III human GBS can be classified by analysis of RDPs into distinct lineages, each of which is comprised of a single serotype. These lineages, called RDP types II-1, II-2, and II-3 and RDP types III-1, III-2, and III-3, can also be distinguished from each other

TABLE 4. STs of serotype II and serotype III bovine strains

Serotype	No. tested	STs[a] (n[b])
II	19	10 (4), **61 (7), 67 (8)**
III	38	19 (1), **17 (3)**, 23 (18), **61 (8), 67 (1)**, 90 (2), 92 (1), 94 (1), 91 (1), 93 (1), 105 (1)

[a] STs in bold are in CC 17.
[b] n, no. of isolates.

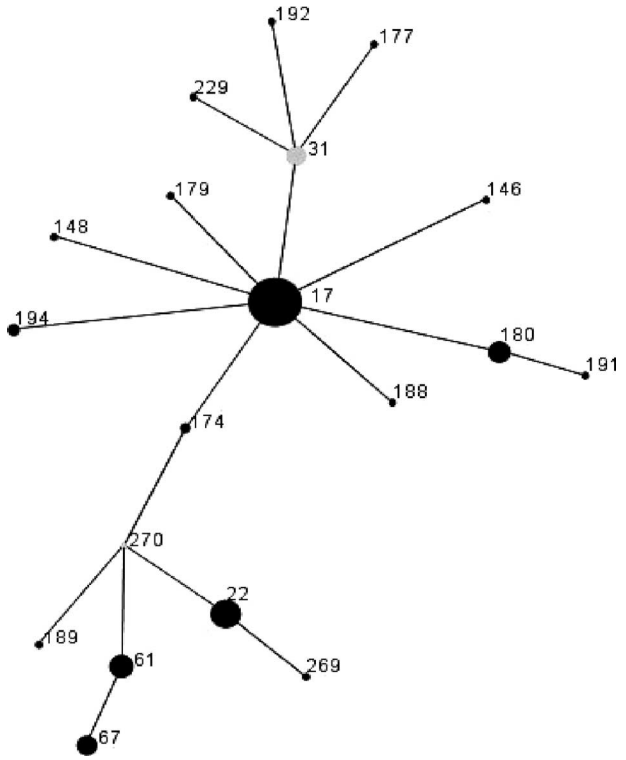

FIG. 3. CC 17 contains both human and bovine GBS isolates. The structure of CC 17 was determined by eBURST analysis of the human and bovine GBS examined in this study.

by analysis of the strains' *infB* alleles and repertoire of selected mobile genetic elements (32). RDP type III-1, III-2, and III-3 GBS have been shown to correspond to CC 23, CC 17, and CC 19 serotype III strains, respectively, but we have not reported the relationship between RDP type II-1, II-2, and II-3 GBS and CC 17 and CC 19 serotype II GBS (16, 32). Analysis of serotype II GBS with known RDP types demonstrated that RDP type II-1 strains are ST-28 (CC 19), RDP type II-2 strains are ST-22 (CC 17), and RDP type II-3 strains are ST-19 (CC 19). The CC 17 serotype II GBS from the current sample also have the *infB* allele and mobile genetic elements characteristic of RDP type II-2 GBS, while CC 19 serotype II GBS have the *infB* allele and mobile genetic elements characteristic of RDP types II-1 and II-3 (Table 5). It is interesting to note that the ST-19 serotype II (RDP type II-3) strains have an insertion sequence genotype that is identical to that of ST-28 serotype II strains rather than to that of ST-19 serotype III strains, suggesting that the serotype II strains in CC 19 more closely resemble each other than they do CC 19 serotype III GBS.

DISCUSSION

The results of this study demonstrate that serotype Ia, Ib, II, III, and V GBS that caused EOD in these six centers from 1995 to 1999 were almost entirely from the same five CCs which predominated among colonizing strains. GBS from these CCs have been shown to cause the majority of neonatal GBS infections in Sweden, the United Kingdom, and Israel (4, 15, 23).

CC 23 serotype Ia GBS, which corresponds to RDP type Ia-2

TABLE 5. Mobile genetic elements and *infB* alleles of human CC 17 and CC 19 GBS

CC	Serotype	No. of isolates	Insertion site genotype			*infB* allele	RDP type[a]
			AW-10	*scpB-lmb*	*hylB*		
17	II	21	IS*1563*-GBSi1	IS*1548*	No insert	A	II-2 (ST-22)
	II	1	No insert	IS*1548*	No insert	A	
	III	90	GBSi1	GBSi1	No insert	C	III-3
	III	1	IS*1563*-GBSi1[b]	IS*1548*	No insert	A	
	III	1	No insert	No insert	No insert	C	
19	II	23	No insert	GBSi1	No insert	A[c]	II-1 (ST-28)
	II						II-3 (ST-19)
	III	122	No insert	IS*1548*	IS*1548*	A[d]	III-2
	III	1	No insert	IS*1548*	No insert	A	

[a] The RDP types and STs of the corresponding serotype II and III strains from reference 32 are shown.
[b] The single serotype III strain with the IS*1563*-GBSi1 insert in AW-10 and IS*1548* in *scpB-lmb* (thus more closely resembling serotype II ST-22 strains) is ST-189.
[c] A single strain had the *infB* B allele, which varies from the A allele by one nucleotide substitution.
[d] A single strains had a novel *infB* allele, which varies from the A allele by one nucleotide substitution.

(32), was also the most predominant serotype Ia GBS lineage isolated from infected neonates in Sweden, the United Kingdom, and Israel (4, 15, 23). The closely related ST-24 serotype Ia strains accounted for 6% of invasive serotype Ia isolates in this study. Together, these data indicate that CC 23 and the closely related ST-24 serotype Ia GBS comprise the most common lineage of serotype Ia GBS associated with invasive disease in human neonates in developed countries. This report also demonstrates that CC 23 strains predominate among serotype Ia strains that colonize human neonates.

Serotype V GBS emerged in the 1990s and have become established globally as an important cause of GBS infection in both neonates and adults. This study, along with previous studies, indicates that these serotype V GBS are clonal and are from CC 1 (4, 5, 15, 16, 23). While colonizing serotype V strains in this study were also occasionally found in a variety of other lineages, the only other invasive serotype V strain in this study was ST-26, an ST related to a bovine serotype V ST-256 strain isolated from a bovine udder in Brazil (27). CC 1 is comprised of the smallest number of STs of the major CCs associated with invasive disease in this study, with 93% of the CC 1 strains having a single ST (ST-1), suggesting that the diversity of STs within a CC may reflect how recently an invasive clone arose in the human population. If so, CC 23 may also be a relatively new human clone, since CC 23 contains a relatively small number of STs and is largely comprised of a single ST, although the small number of STs may represent a sampling bias. CC 1 also contains strains of multiple other serotypes, including serotypes Ib, II, III, IV, VI, and VIII. Serotype Ib, II, and IV GBS from CC 1 have been associated with invasive disease in humans (4, 15, 16, 23).

Invasive neonatal serotype Ib strains in this study are almost entirely from a group of two closely related clones that together comprise a single CC, here called CC 12. This CC has also been referred to as CC 9 or CC 10 in descriptions of serotype Ib GBS isolated in other geographic locations, because the CC frequently contains invasive ST-9 and ST-10 serotype Ib GBS, STs which are not represented in the CC 12 strains in this study (4, 15, 16, 23). The reason for the different geographic distribution of STs of serotype Ib GBS from this clonal cluster is not known, and this contrasts with the worldwide distribution of other GBS STs.

Most serotype II GBS in this sample were either ST-22, ST-19, or ST-28. ST-19 and ST-28 strains clearly cluster within CC 19, but this is the first time that ST-22 GBS have been assigned to CC 17 (3, 4, 15, 16, 23). Clustering of ST-22 GBS into CC 17 with the bovine ST-61 GBS and related bovine GBS is consistent with the previous assertion that human ST-17 GBS arose from bovine GBS with STs similar to ST-61 (3). The genetic relationship between human and bovine CC 17 strains is interesting, but it is worth noting that this is not a unique relationship among bovine and human GBS. As shown in Table 4, ST-10 and ST-23 GBS are also commonly isolated from bovine sources.

Analysis of the *inf*B alleles and mobile genetic element repertoire also indicates that CC 17 serotype II strains and CC 17 serotype III strains, as well as CC 19 serotype II strains and CC 19 serotype III strains, are distinct from each other and are each relatively homogeneous lineages. The latter assertion is supported by the ability of RDP analysis to separate these strains into distinct RDP types, previously designated RDP types II-2, III-3, II-1 and II-3, and III-2, respectively (32). The observation that GBS of different serotypes that share a common ST can be quite genetically divergent has been recently demonstrated at the genomic level (33). Despite the genetic divergence indicated by ST distribution, RDP analysis, repertoire of mobile genetic elements, and *infB* alleles, human serotype II and III GBS in CC 17 exhibit similar clinical behavior in their apparent tendency to be more commonly associated with bloodstream infection than surface colonization in human neonates (22). These data suggest that intrinsic genetic properties that affect the ability to colonize and invade neonates have persisted in CC 17 strains of both serotypes despite other genetic changes that differentiate these lineages. However, it is unclear why CC 17 serotype II GBS appear to be more commonly associated with invasive disease in this study than in studies from other countries (4, 15, 16, 23).

Although the majority of invasive and colonizing isolates in this study were found in five CCs, seven rare clones were found in both invasive and colonizing isolates. Interestingly, 22 additional rare clones found among 38 colonizing isolates were not in any invasive isolates. Several new pathogenic clones of GBS have arisen since GBS was first identified as a neonatal pathogen (most notably serotype V GBS from CC 1), raising the

possibility that the rare clones identified in this investigation are the first sightings of newly arisen pathogenic GBS or the remnants of previously more widely spread clones.

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