Genetic Differences Between Invasive and Noninvasive Neonatal Group B Streptococcal Isolates

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Background: *Streptococcus agalactiae*, also known as group B streptococcus (GBS), is the most common cause of neonatal sepsis and meningitis. To improve our understanding of the pathogenesis of neonatal GBS sepsis, better knowledge of clonal relatedness and diversity among invasive and noninvasive GBS isolates is critical.

Methods: In a Germany-based study, invasive neonatal GBS isolates were compared with noninvasive isolates from neonates in whom sepsis was suspected, but whose blood cultures were sterile. The comparison was conducted by means of pulsed-field gel electrophoresis and surface protein gene profiling. In addition, multilocus sequence typing was performed on invasive and noninvasive isolates of the most frequent invasive serotype III.

Results: Pulsed-field gel electrophoresis analysis of noninvasive GBS showed a remarkably more diverse fingerprinting pattern than that of invasive isolates. In contrast to invasive strains, noninvasive isolates did not show any clustering. Surface protein gene profiling also showed significantly different distribution patterns between the 2 panels of isolates. Multilocus sequence typing of invasive and noninvasive serotype III isolates revealed the same clonal complexes, but displayed different sequence types (ST); ST-17 was most common (68.6%) among invasive strains, whereas ST-389 (clonal complex-19) was predominant among noninvasive strains (47.8%).

Conclusions: Our results illustrate a large molecular diversity among neonatal noninvasive GBS strains. Invasive strains, however, represent only a small proportion of the noninvasive GBS population. These findings suggest a selection process that prefers more virulent strains during invasion.

Key Words: *Streptococcus agalactiae*, group B streptococcus (GBS), neonatal, invasive disease, molecular epidemiology

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Streptococcus agalactiae (group B streptococcus [GBS]) is the leading cause of neonatal sepsis and meningitis.[1] GBS infection is classified as either early-onset (EOD, age 0–6 d) or late-onset disease (LOD, age 7–90 d). EOD is associated with maternal colonization and vertical intrapartum GBS transmission.[2] LOD is less well understood and may result from community, nosocomial, or maternal acquisition of GBS.[3]

Transmission and colonization of the mucosal surfaces of the neonate is most likely to be first step toward invasive disease. Colonizing or noninvasive strains which do not enter the blood stream or—at least—are not detectable in blood or cerebrospinal fluid (CSF) cultures and strains causing invasive disease might be genetically different. Therefore, it seems of interest to study the molecular epidemiology of invasive and noninvasive GBS strains and their genetic relatedness.[4] In addition, the identification of more virulent strains might offer focused opportunities to design more specific vaccines for strains with the particular properties that facilitate invasiveness in humans.

Commonly used typing techniques include pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). Serotype distribution and PFGE profiles of 296 invasive neonatal GBS isolates from a nationwide German surveillance study have been described recently.[5,6] To disclose possible differences between invasive and noninvasive strains in PFGE clusters, we additionally conducted PFGE on a panel of 171 neonatal noninvasive strains.

Serotype III is the most frequent serotype causing invasive disease, and molecular typing patterns of GBS have been most intensively studied in this serotype. For this reason, we decided to perform MLST on all serotype III (188 invasive and 46 noninvasive) isolates of both the panels. MLST is currently the most reliable method for the unambiguous comparison of strains worldwide. Moreover, it has been suggested that some strains, for example, MLST sequence type (ST)-17, are associated with enhanced invasiveness.[7]

As an additional typing approach, surface protein genes of both the panels, including invasive and noninvasive strains, were analyzed by polymerase chain reaction (PCR).[8] Surface proteins such as the alpha-C protein, Rib, Alp2, Alp3, Alp4, and the epsilon protein are thought to be involved in pathogenesis of GBS disease.[9–11]

MATERIALS AND METHODS

Bacterial Isolates

From April 2001 through March 2003, active Germany-wide surveillance for invasive GBS infections in neonates up to the age of 3 months was independently performed in all German pediatric hospitals and affiliated microbiological laboratories, as described previously.[5] A total of 296 invasive neonatal isolates were collected.

During the same time period, noninvasive neonatal strains were collected in 4 German cities from 4 different regions (West, South-West, North, and South-East). In this instance, a total of 90 isolates were collected. These noninvasive strains were isolated from newborns within the first 24 hours of life who had been hospitalized for suspicion of sepsis, but in whom blood cultures remained sterile. Additional 81 isolates from nonsterile sites of newborns with suspected EOD, which had been mistakenly sent to

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TABLE 1. Relationship Between Serotypes and Main PFGE Groups Within GBS Neonatal Invasive and Noninvasive Strains

		No. and Percentage of Isolates per Serotype					
		Ia	Ib	II	III	V	IV/NT
No. invasive strains	288	44 (15%)	14 (5%)	15 (5%)	188 (65%)	22 (8%)	2/3 (1%/1%)
Invasive PFGE group							
A	57	1	0	0	56	0	0
B	28	0	0	0	28	0	0
C	24	20	0	0	4	0	0
D	21	0	1	0	1	18	0/1
E	14	1	10	0	3	0	0
F	14	0	1	2	10	1	0
G	14	0	0	0	14	0	0
No. noninvasive strains	171	24 (14%)	21 (12%)	21 (12%)	52 (30%)	32 (19%)	3/18 (2%/11%)
Noninvasive PFGE group							
D-NI	22	0	1	1	2	17	0/1
C-NI	10	1	3	0	2	1	1/2
C'-NI*	9	5	0	0	2	0	0/2
E-NI	8	7	0	0	0	0	0/1

*There was only a small (visual) difference between groups C-NI and C'-NI, but both groups were separated automatically by the GelCompar II software.
PFGE indicates pulsed-field gel electrophoresis.

the central laboratory in Freiburg by the various participating hospitals from all parts of Germany (as contribution to the earlier mentioned surveillance study) were also included. By definition, these isolates were cultured from newborns in whom "true" invasive disease had been excluded by negative blood and CSF cultures. In total, 171 noninvasive neonatal GBS isolates were available. Serotyping was performed as described previously.[5]

Pulsed-field Gel Electrophoresis

PFGE analysis was performed as previously described.[6] In brief, genomic DNA of all GBS isolates was prepared and subsequently, digested using the restriction endonuclease SmaI (New England BioLabs, Frankfurt, Germany). After digestion, PFGE was performed on the CHEF-DR II apparatus (Bio-Rad Laboratories, Richmond, CA). The obtained restriction digest patterns were visually analyzed following the Tenover criteria, and a second computer-assisted analysis was performed using the GelCompar II software (Applied Maths, Austin, TX). Isolates were considered to be related (assembled within the same PFGE group), if they showed a maximum variation by 1 to 3 bands.[6]

Determination of Surface Protein Genes

Analysis of surface protein genes was performed according to the method described by Creti et al.[8]

Multilocus Sequence Typing

Of the serotype III, 188 invasive and 46 noninvasive neonatal strains were available and subjected to MLST; 6 out 52 serotype III noninvasive strains unfortunately could not be recultured. The detailed MLST protocol has been published recently.[12] Allele and sequence type assignment were made using the GBS MLST database.[7]

Statistical Analysis

Serotype and surface protein distributions were compared with the χ^2 test.

RESULTS

Serotyping and PFGE Results of Invasive and Noninvasive GBS Strains

The PFGE results of the invasive strains have been described recently.[6] In summary, 7 major groups were detected among 288 (out of 296) isolates that were able to be digested using the restriction endonuclease *Sma*I. These 7 groups comprised 60% of all invasive isolates. Four groups were either dominated by or exclusively formed by serotype III strains (Table 1).

Serotype distribution of the noninvasive strains demonstrated that serotype III was most prevalent (30%), followed by serotypes V (19%), and Ia (14%). Serotype III was the most common, but the percentages differed significantly between invasive (65%) and noninvasive groups (30%), respectively ($P < 0.001$). When comparing only EOD serotype III and noninvasive serotype III isolates, the difference was also significant (58% vs. 30%, $P < 0.001$).

The extent of genetic diversity among noninvasive isolates (147 patterns for 171 isolates) as well as among invasive isolates (185 patterns for 288 isolates) likewise differed considerably. PFGE patterns within serotypes varied strongly, and genetic diversity among noninvasive strains of the same serotype was greater than among invasive isolates. At a level of 80% similarity, 4 major PFGE groups among noninvasive strains were evident, mainly representing serotype Ia and V strains (Table 1). The PFGE profiles of these groups were similar (at a level of at least 80% similarity) to the corresponding profiles of invasive strains and were named, for example, PFGE group C-NI (NI for noninvasive). The major PFGE profiles were mostly comprised of isolates of the same serotype. Nevertheless, different PFGE profiles could be designated to each serotype.

In contrast to the invasive strains, noninvasive serotype III strains did not cluster. None of the 4 major groups of noninvasive strains was dominated by serotype III isolates (Table 1).

MLST Sequence Types of Invasive and Noninvasive Serotype III Isolates

In all, 188 invasive and 46 noninvasive serotype III isolates were subjected to MLST (Table 2). The invasive isolates could be classified in 18 known ST groups. Seven were variants of different ST described previously, and 1 strain had an allele combination which had not been described previously (ST-454, −455, −456, −457, −465, −466). Among invasive isolates, 2 major genetic lineages were detected; 129 (68.6%) of all invasive serotype III strains belonged to ST-17 and 27 strains (14.4%) to ST-19. Other STs were less common and represented 1 to 5 (ie, ST-23) isolates.

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TABLE 2. Sequence Types and Clonal Complexes of All Serotype III Strains Subjected to MLST

Invasive Strains (n = 188)								Noninvasive Strains (n = 46)			
Early-onset Disease (n = 96)				Late-onset Disease (n = 89)							
ST	n	CC	n	ST	n	CC	n	ST	n	CC	n
4	1	4	1					186	1	1	2
8	1	8	2	8	1	8	1	370	1		
17	61	17	64	17	67	17	71	8	1	8	1
19	18	19	22	19	8	19	12	17	11	17	14
23	5	23	6	23	1	23	2	147	2		
27	1			27	1			148	1		
52	1			171	2			389	22	19	23
106	2			176	1			182	1		
109	1			233	2			23	2	23	3
147	1			440	1			24	1		
182	1			455	1			200	2		
188	1			456	2			7	1		
389	1			457	1						
454	1			465	1						

Unknown onset of 3 invasive strains with ST-17, -19, -408, respectively.
ST indicates sequence type; CC, clonal complex.

Sequence types were grouped into clonal complexes (CC) on the basis of sharing a threshold level of allelic identity with a central ST in the group (typically 5 or 6 identical loci). In total, 136 isolates belonged to CC-17, 35 to CC-19, and 8 to CC-23.

ST distribution among noninvasive isolates was different; ST-389, a single locus variant of ST-19, was most common (n = 22; 47.8%). In contrast, ST-389 was identified only in a single case among the invasive strains. Strains with the original ST-19 were not identified. ST-17 was found in 11 noninvasive isolates (23.9%). Thus, ST-17 was detected significantly more often in invasive serotype III isolates ($P < 0.001$), whereas ST-389 was nearly exclusively found in noninvasive serotype III isolates ($P < 0.001$). A graphic representation of the results is shown in Fig., Supplemental Digital Content 1, http://links.lww.com/INF/A900.

A significant correlation between ST-17 serotype III isolates and onset of infection could not be determined (63.5% and 70.5%, respectively). ST-19 was more often found among isolates causing EOD, without reaching statistical significance.

Of the 188 invasive serotype III strains, 108 strains clustered in the major 4 PFGE groups A, B, F, and G. ST-17 was the prevailing ST in these 4 major "serotype III" PFGE groups (Table 3). PFGE groups A, B, and G were exclusively formed by ST-17 isolates (except for one serotype Ia isolate). In PFGE group F (70% serotype III), mainly ST-19 isolates were found. PFGE group C was dominated by serotype Ia, but also 4 serotype III strains were identified. Three of them expressed ST-23, an ST usually associated with serotype Ia. Therefore, genetic relatedness of serotype III and Ia strains in this PFGE group could be confirmed.

Surface Protein Gene Profile

Genes of major surface proteins genes (SPG) of GBS were determined by multiplex PCR. A PCR product was detected in 88% of the invasive strains. None of the strains had more than one SPG detected. The *rib*-gene was most prevalent among invasive isolates (48%), followed by epsilon (12%), alp 2/3 (11%), and alpha-C protein gene (10%). The *alp4*-gene was not detected. Specific relationships between SPG and serotypes in invasive isolates were noticed. The epsilon protein gene predominantly was found in serotype Ia (65%, $P < 0.001$), whereas the alpha-C protein gene most frequently was detected among serotype Ib (>70%) and II (40%) strains. Detection of the rib protein gene was associated with serotype III (75%, $P < 0.001$), but was also found in 50% of serotype II strains. The alp2/3-genes were primarily detected in serotype V isolates (65%). Approximately 10% of strains contained no SPG.

The distribution of SPG between invasive and noninvasive strains varied (Fig., Supplemental Digital Content 2, http://links.lww.com/INF/A901). Comparable to the invasive strains, the epsilon protein gene was predominantly found in serotype Ia isolates (50%). The alpha-C protein gene mainly was found among serotype Ib (71%). The percentage of alpha-C protein gene positive isolates among noninvasive serotype II strains was lower than that among invasive strains (28% vs. 40%). The *rib* gene was associated with serotype III isolates but to a lesser extent (52%). Comparable to the invasive isolates, the *rib* gene was found in 43% of serotype II isolates. The alp2/3-genes were primarily detected in serotype V isolates (50%). Nearly half of the noninvasive isolates without SPG belonged to serotype III (Table 3).

DISCUSSION

GBS remains a major pathogen of sepsis and meningitis in newborns. Our understanding of the pathogenesis of GBS disease may be improved by a more comprehensive understanding of the genetic structures of invasive and noninvasive neonatal GBS isolates. Because transmission of the pathogen from the mother and subsequent colonization of the newborn is most likely to be the first step toward invasive disease, studying the molecular epidemiology of neonatal noninvasive GBS strains and comparing their molecular pattern to that of isolates causing invasive disease appears to be of major importance.

PFGE results of the invasive isolates have been recently described.[6] Seven major PFGE groups were detected. Three groups (ie, groups A, B, G) were nearly exclusively comprised of serotype III isolates. Only one isolate in group A displayed serotype Ia. In the fourth group (group F), serotype III was detected in nearly 70%, but additional serotypes were also found. The other major groups were dominated by serotype Ia, Ib, and V, respectively (Table 1).[6] In contrast to the results of other groups, strains with identical macrorestriction patterns did not always express the same serotype.[13,14] Furthermore, any serotype found in this study could also be detected in any of the different PFGE groups. This reflects the observations described by Gherardi et al for Italian isolates.[15] A possible explanation may be horizontal gene transfer, driven by the host immune response to GBS colonization.[16]

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TABLE 3. Relationship Between PFGE, Serotypes, Surface Protein Genes, Sequence Types and Clonal Complexes Within GBS Neonatal Invasive and Noninvasive Strains

PFGE Group	No. Isolates	Invasive/ Noninvasive Strains	Serotypes (No. Strains)	Surface Protein Gene (No. Strains)	ST of Serotype III Strains (No. Strains)	Clonal Complex (Number), [Degree of Relatedness]
A	57	Invasive	III (n = 56), Ia (n = 1)	Rib (n = 43), no SPG (n = 7), alp 2/3 (n = 5), alpha/epsilon (n = 1 each)	17 (n = 53), 19, 109, new ST (n = 1 each)	17 (n = 54), 19 (n = 1) [SLV]
B	28	Invasive	III (n = 28)	Rib (n = 22), no SPG (n = 4), alpha/epsilon (n = 1 each)	17 (n = 24), new ST/171 (n = 1 each)*	17 (n = 26), [SLV]
C	24	Invasive	Ia (n = 20), III (n = 4)	Epsilon (n = 14), alpha/no SPG n = (4 each), rib/alp2/3 (n = 1 each)	19 (n = 1), 23 (n = 3)	19, 23 [SLV]
D	21	Invasive	V (n = 18), Ib/III/NT (n = 1 each)	Alp2/3 (n = 14), rib (n = 4), no SPG (n = 2), alpha (n = 1)	17 (n = 1)	17
E	14	Invasive	Ib (n = 10), III (n = 3), Ia (n = 1)	Alpha (n = 12), rib/no SPG (n = 1 each)	8 (n = 2), new/SLV8 (n = 1)	8 (n = 3) [SLV]
F	14	Invasive	III (n = 10), II (n = 2), Ib/V (n = 1 each)	Rib (n = 9), no SPG (n = 3), alp/alpha (n = 1 each)	19 (n = 4), 17 (n = 2), 27/ 389/new ST (n = 1 each)	19 (n = 7), 17 (n = 2)
G	14	Invasive	III (n = 14)	Rib (n = 7), no SPG (n = 4), alp 2/3 (n = 2), epsilon (n = 1)	17 (n = 13), 188 (n = 1)	17 (n = 14), [SLV]
D-NI	22	Noninvasive	V (n = 17), III (n = 2), Ib/II/NT (n = 1 each)	Alp2/3 (n = 13), rib (n = 4), no SPG (n = 3), alpha/epsilon (n = 1 each)	186 (n = 1), 389 (n = 1)	1, 19 [SLV]
C-NI	10	Noninvasive	Ib (n = 3), III/NT (n = 2 each), Ia/IV/ V (n = 1 each)	Alpha (n = 8), rib (v1), no SPG (n = 1)	8 (n = 1), 200 (n = 1)	200 [SLV 10]
C'-NI	9	Noninvasive	Ia (n = 5), III/NT (n = 2 each)	Epsilon (n = 5), rib (n = 3), alpha (n = 1)	17 (n = 1), 24 (n = 1)	17, 23 [DLV]
E-NI	8	Noninvasive	Ia (n = 7), NT (n = 1)	Alpha (n = 4), epsilon (n = 3) no SPG (n = 1)	—	—

*Two strains were not available for MLST analysis.
SPG indicates surface protein gene; ST, sequence type; SLV, single locus variant; DLV, double locus variant.

To disclose possible differences, noninvasive neonatal isolates collected during the same time period were also subjected to PFGE, MLST, and surface protein gene profiling. In contrast to invasive strains, the PFGE profiles of noninvasive isolates were more diverse. Still, some isolates of serotypes V and Ia (groups D-NI, C'-NI, and E-NI, Table 1) showed less diversity and clustered in major groups, which were similar or equal to some of the invasive PFGE groups. For all other serotypes, nearly one single pattern per strain was found. Therefore, we are unable to confirm the results of other investigators who found that, for example, all serotype V isolates showed a distinct PFGE profile.[17]

Serotype III was also the most prevalent serotype among noninvasive isolates, although to a significantly lesser degree. The noninvasive serotype III strains did not cluster in specific PFGE groups. This observation implies that although belonging to the identical serotype, invasive serotype III strains differ from noninvasive strains. From a different point of view, only a very small proportion of noninvasive isolates clearly have the potential capacity to establish invasive disease. Thus, additional factors beyond capsular polysaccharides seem to contribute to enhanced virulence and invasiveness. Finally, remarkable diversity of serotypes was found among strains with identical macrorestriction patterns.

Surface proteins such as the alpha-C protein, Rib, Alp2, Alp3, Alp4, and the epsilon protein are thought to be involved in pathogenesis of GBS disease. They belong to a family of surface proteins with internal tandem repeats and are considered to be potential virulence factors promoting escape mechanisms from the immune response.[9,10] Their detection rates among GBS isolates seem to vary over time and geographic location.[18,19] Associations between serotypes and surface proteins have been described.[17,20–23] Interestingly, the order of protein genes detected differed between invasive and noninvasive strains (Fig., Supplemental Digital Content 2, http://links.lww.com/INF/A901).

The association of the *rib* gene and serotype III was confirmed.[22,24] Interestingly, the percentages of *rib*-positive isolates within the 2 panels of serotype III strains varied considerably; among invasive isolates, *rib* was detected in 75% and among noninvasive strains in 52% of cases. This finding confirms recent results, suggesting that *rib* plays a role in invasive disease.[25] The complete absence of SPG has been reported to occur rarely in GBS.[9] This contradicts our results where approximately 20% of the GBS isolates were tested negative for any SPG.

Serotype III has been consistently described to be associated with neonatal sepsis and meningitis.[12,18] Likewise, 65% of invasive disease in our study was caused by serotype III isolates.[5] MLST has become the standard method to determine the genetic structure of GBS. To disclose possible molecular differences between invasive and noninvasive serotype III isolates, all serotype III strains were examined by MLST. The MLST patterns found correlated partly to patterns of neonatal strains from other countries. It has been described that strains belonging to certain clonal groups, such as ST-1, ST-17, ST-19, and ST-23, are main causes for human infections.[16,26] ST-1 is associated with serotype V isolates. Therefore, it is not surprising that only one strain of CC-1 was detected here. ST-23 is associated with serotype Ia. This genetic lineage has been described to be one of the most important

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in causing human infections.[4,12,16] We identified few ST-23 strains among serotype III isolates. Nevertheless, these ST-23 strains were found in a PFGE group dominated by serotype Ia. Therefore, MLST results complemented PFGE results, and genetic relatedness was verified, despite expression of different capsular serotype.

ST-17 is widespread. Isolates belonging to CC-17 are associated with neonatal sepsis and meningitis. This highlights that ST-17 is well adapted in successfully escaping the host immune response. Alternatively, it may possess virulence factors that are prerequisites for invasiveness.[12,16] To date, only ST-17 which appears to be of bovine origin has been found in serotype III isolates. Manning et al found high percentages of ST-17 and -19 isolates in LOD.[27] In our study, ST-17 was equally distributed between EOD and LOD, whereas ST-19 was more frequently found in EOD than in LOD. Neither ST-17 nor -19 could be associated with CSF infections.

Among serotype III, the same CCs were found among noninvasive and invasive strains. Nevertheless, different dominating STs were identified. Invasive serotype III strains significantly were associated with ST-17 and CC-17 ($P < 0.001$), whereas ST-389 (a single locus variant of ST-19) dominated among colonizing strains (47.8%, $P < 0.001$). The ST-389 strains were collected from different regions of Germany. Therefore, we are able to exclude the observed clonality having been related to a local outbreak. This reinforces our assumption that the predominance of ST-17 is not related to an overrepresentation of serotype III strains, but rather due to an enhanced virulence of this ST. Affiliation with CC-17 seems to characterize strains with enhanced invasiveness independent of differences in ST, restriction digest pattern analysis, or presence of mobile genetic elements.[28] These results partially are reflected in the PFGE results, where serotype III strains belonging to different PFGE groups could be assigned to different STs.

Nevertheless, the characterization of isolates in this study is a description of a time- and location-specific situation, and most probably, the depiction will not remain stable. Considerable fluctuations in the GBS pool have been described, and new clones able to cause invasive disease have appeared.[4,29] Additionally, the occurrence of rare clones/ST has been documented.[30] We also found ST singletons. Further observation of ST distribution is necessary to determine whether these single STs are the first signs of new arising ST or else only temporary appearances. In this sense, the predominance of ST-389 among the noninvasive serotype III strains may be an indicator of a shift toward a new ST within CC-19. In conclusion, our data show that invasive strains represent only a small proportion of the noninvasive GBS population, which argues in favor of a selection process for more virulent strains during invasion.

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