Microarray Analysis of Group B Streptococci Causing Invasive Neonatal Early- and Late-onset Infection

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Background: Group B Streptococcus is the leading cause of meningitis and sepsis in newborns. Until now, there is no data of fast and simple typing of group B Streptococcus virulence factors using a genetic microarray and comparing these data to clinical manifestations.

Methods: A prospective active surveillance study was conducted via 2 independent and nationwide reporting systems, the German Pediatric Surveillance Unit (ESPED) and the Laboratory Sentinel Group at Robert Koch-Institute. Surveillance was performed between 2001 and 2003 and between 2008 and 2010. Typing of virulence factors, serotypes, pilus islands and alpha-like proteins was done by means of a newly developed microarray method.

Results: We evaluated 475 isolates of invasive neonatal infections. Predominant virulence factors were serotype III (63%), pilus island 2b and pilus island 1 (50%) and alp rib (64%) (alp - alpha-like protein, rib -resistance to proteases, immunity, group B). There was no significant change over time or geographically within Germany. Serotype III, pilus island 2b + 1 and alp rib showed significant associations with late-onset disease and meningitis, whereas alp 5 had a significant association with early-onset disease. Based on serotypes, pilus islands and alpha-like proteins, it was possible to cluster 86% of all isolates into 5 genetic groups.

Conclusions: The molecular epidemiology of a large collection of invasive neonatal infections showed similar distributions, as shown in smaller cohorts before. The microarray used proved to be a fast and reliable technique. Using this new tool, we were able to cluster the isolates according to their virulence factors. The clusters showed a better association with clinical data than single virulence factors.

Key Words: group B Streptococcus, microarray, serotype, Pilus Island, alpha-like protein

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Group B Streptococcus (GBS) is well-known as a leading cause of sepsis and meningitis during the first 90 days of life.[1–3] With case-fatality ratios of 9.6% worldwide[4] and 4% in Germany,[5] GBS is significantly linked to morbidity and mortality among newborns and infants.

Neonatal GBS disease presents as either early-onset disease (EOD, age 0–6 days) or late-onset disease (LOD, age 7–90 days)—both with different transmission pathways, clinical presentations, sequelae and prognosis.[5] Newborns with EOD become infected vertically. In 90% of cases, onset of disease occurs during the first 48 hours after birth.[6] GBS colonizes 7%–36% of pregnant women in Europe.[7] With LOD, transmission is vertical or horizontal.[8,9] In contrast to EOD, LOD more often is accompanied by meningitis, and more frequently leads to residual impairments.[5]

Numerous studies on the molecular characteristics of GBS have been performed to improve understanding of pathogenesis and epidemiology. Known virulence factors include alpha-like proteins,[10–13] pilus proteins[14–19] and capsule polysaccharides.[4,20–24] Typing techniques included pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST). One newly-evolved method is the use of a DNA microarray that targets GBS genes coding for virulence markers, surface proteins, etc.

MLST is a typing method using seven housekeeping genes, each with different possible alleles. Depending upon the alleles, one allelic profile or sequence type (ST) is assigned. Similar sequence types are categorized into one clonal complex. Using the PFGE technique, endonucleases digest DNA (eg, SmaI) into fragments that get separated by gel electrophoresis using changing voltage. The results show a characteristic DNA band pattern, which then becomes interpreted as genome-wide DNA fingerprinting. In contrast to these 2 techniques, the microarray is faster and less cumbersome; it is highly reproducible and covers larger parts of the genome (ie, 200+ genes). Due to its pan-genomic coverage and its independence from single nucleotide polymorphism, it may even quantify genetic differences more realistically.[8]

MATERIALS AND METHODS

Case Definition

Neonatal and invasive infection cases were defined as isolation of GBS from blood or cerebrospinal fluid in infants ≤ 90 days of age. Disease onset during days 0–6 was described as "early" (EOD), and onset during days 7–90 was described as "late" (LOD). The case definition was identical for the 2 surveillance periods.

Case Finding

Surveillance was performed in Germany from April 1, 2001, to March 31, 2003 and from January 1, 2008, to December 31, 2010. The prospective, active surveillance study was conducted through 2 independent, nationwide reporting systems. The German Pediatric Surveillance Unit (ESPED) sent pediatric departments monthly inquiries requesting reports of invasive GBS infection. Reporting hospitals received a standardized questionnaire to collect details about pregnancy, birth, the infant's clinical history, diagnosis, treatment and outcome. The Laboratory Sentinel Group at the Robert Koch-Institute sent the microbiologic laboratories serving pediatric hospitals monthly inquiries as well. All isolates, along with accompanying metadata, such as postal code and month of birth, were collected through this surveillance system. The GBS isolates were sent to the Department of Pediatrics and Adolescent Medicine, University of Freiburg, Germany, for further analysis. They were stored at −80°C.

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Ethics Statement

Isolates were obtained anonymously. Ethics approval was provided by the Ethics Committee of the Medical Faculty of the University of Freiburg, Germany.

Preparation of Genomic DNA

Genomic DNA preparation was done according to standard methods.[25]

Amplification Reaction

According to the HybridisationsPlus Kit (Alere), 5 µL purified DNA reacted with 3.9 µL Labelling Buffer (Alere), 0.1 µL Labelling Enzyme (Alere) and 1 µL mix of primer (Alere). Polymerase chain reaction (PCR) amplification was composed of an initial denaturation step with 96°C for 300 seconds, with 45 cycles (20 seconds at 60°C, 30 seconds at 72°C and 20 seconds at 96°C) and final storage at 4°C.

Microarray

The microarray from Abbott/Alere Technologies (Jena, Germany) has been specially customized for GBS. It consists of 213 probes that cover genes associated with virulence factors, such as capsule types, pilus proteins and alpha-like proteins, as well as additional virulence genes, for example, ones associated with typing markers, resistance genes and metabolic functions.[25] The procedure was applied according to the manufacturer guidelines (Abbott/Alere Technologies, Jena, Germany). Reagents supplied with the kit were used. Arrays were analyzed using ArrayMate (Alere Technologies, Jena, Germany), a device that scans the array and determines signal intensities of the probes in relation to the local background. Breakpoints are assigned based upon signal intensities of positive and staining controls.[25]

Validation of the Microarray

The latex agglutination test for GBS (ImmuLexTM Strep-N kit, Statens Serum Institute, Copenhagen, Denmark) was used for serotyping. The Etest and disk diffusion tests were employed to phenotypically prove erythromycin resistance. Genetic testing for capsular serotypes by PCR was not performed.

Statistical Analysis

Analyses were conducted with IBM SPSS Statistics 22 (Armonk). The χ^2 test was employed to detect significant associations. A P-value < 0.05 was considered statistically significant. Multivariate logistic regression was done with the algorithm of "Enter," and the cutoff for classification was set at 0.5 for the analyses with EOD/LOD and at 0.44 for the calculation with and/or absence of meningitis.

RESULTS

Bacterial Isolates

Overall, 475 GBS isolates were collected: 277 in the first surveillance period (2001–2003) and 198 in the second (2008–2010). ESPED documents were available for 234 isolates and Robert Koch-Institute documents were for 343 isolates. Data regarding EOD/LOD existed for 349 cases.

Microarray Validation

The microarray detected the various predefined virulence factors in 98% of the isolates. In 92% of isolates (n = 437), results were compared with established methods, such as the latex agglutination test for capsule typing. Congruent results were found in 97.5% (426/437 cases). For the remaining 11 isolates, despite multiple attempts, it was impossible to determine the capsule type via latex agglutination. Additionally, phenotypic erythromycin resistance was compared with resistance genes of the microarray. Results were congruent in 94% of the tested 436 isolates (Table 1). Although 5% of isolates were phenotypically resistant, resistance gene *ermA* or *ermB* was negative, and an additional 1% of isolates were found to be phenotypically sensitive but carrying the *ermA* or *ermB* genes. The resistance gene *mefA* was not tested with the microarray (erm - erythromycin resistance methylase).

TABLE 1. Phenotypic and Genotypic Erythromycin Resistance (n = 436)

	ermA and ermB Negative	ermA and ermB Positive	Total Numbers
Phenotypically sensitive	375 (86%)	3 (0.7%)	378
Phenotypically resistant	22 (5%)	36 (8.3%)	58
Total numbers	397	39	436

Molecular Epidemiology

Serotype III accounted for 63% of all isolates (n = 296), followed by serotype Ia (19%, n = 89), V (7%, n = 33), Ib (6%, n = 28), II (3.8%, n = 18), IV (0.6%, n = 3), VI and IX (each 0.2%, n = 1). Six isolates were nontypeable by the microarray. Genes for pilus island 2b combined with pilus island 1 (PI-2b + PI-1) represented 49.8% (n = 234) of the pilus islands. The remaining pilus islands were PI-2a + PI-1 with 29.2% (n = 137), PI-2a with 19.4% (n = 91) and PI-2b with 1.7% (n = 8). Genes for the alpha-like proteins were nontypeable in 15 cases, with 14 of them showing signal intensities close to the breakpoint for *alp 4*. A majority of cases (63,9%, n = 294) were *alp rib* positive. The remaining were *alp 5* with 17% (n = 78), *alp bca* with 10.2 % (n = 47), *alp 3* with 6.7% (n = 31) and *alp 2* with 2.2 % (n = 10).

Spatial or Timely Clustering

Postal code analysis showed no significant association between virulence factors and specific regions of Germany. When comparing the 2001–2003 and 2008–2010 periods, no significant increase or decrease in a single virulence factor was able to be noted.

Clinical Data and Virulence Factors

Certain significant associations between clinical characteristics of the study population and virulence factors were found, as shown in Table 2. The associations of PI-2b + 1 to LOD and of serotype III to LOD and meningitis are displayed in Figures 1–3. Virulence factors were not significantly associated with birth weight, premature birth, gender, therapy with catecholamines (as a surrogate for disease severity) or sequelae at the time of discharge.

TABLE 2. Significant Associations in Multivariate Analysis ($P < 0.05$) Between Virulence Factors and Clinical Characteristics

	Onset of Disease		Meningitis	
	EOD	LOD	No Meningitis	Meningitis
Serotypes	Ib, II	III	Ia	III
Pilus islands	PI-2a + PI-1	PI-2b + PI-1	PI-2a	PI-2b + PI-1
Alpha-like proteins	alp 5	alp rib	alp 5	alp rib

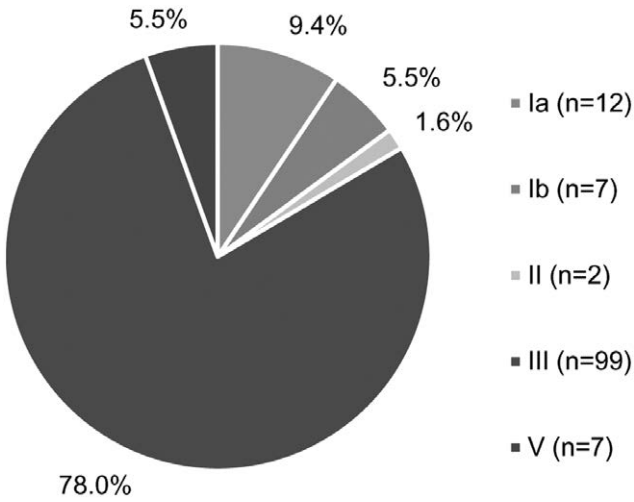

FIGURE 1. Distribution of capsular genotypes among 127 reported cases of meningitis.

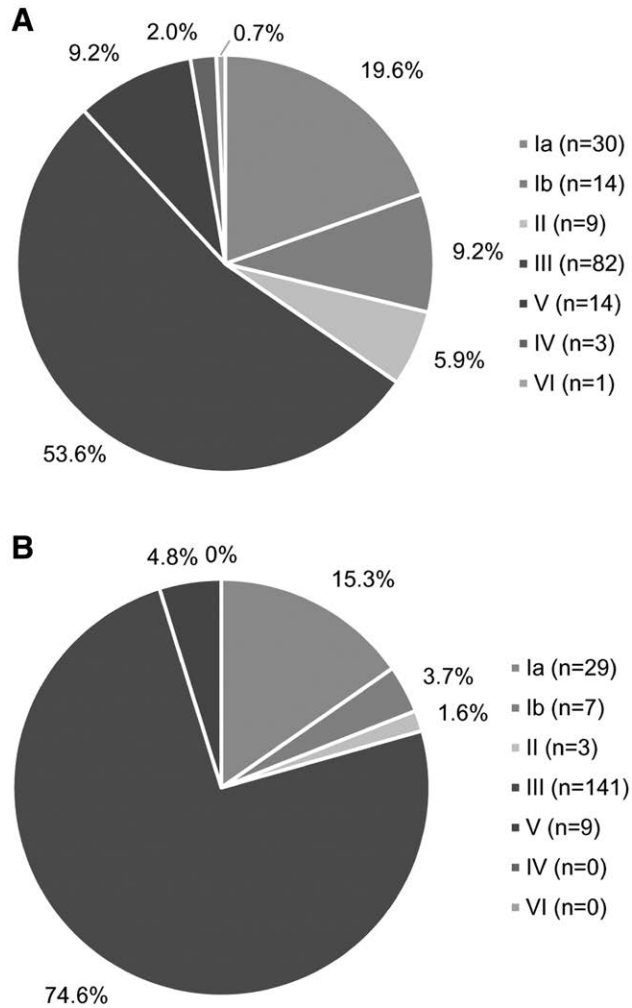

FIGURE 2. Capsular genotype distribution among cases with EOD (n = 153, A) and LOD (n = 189, B).

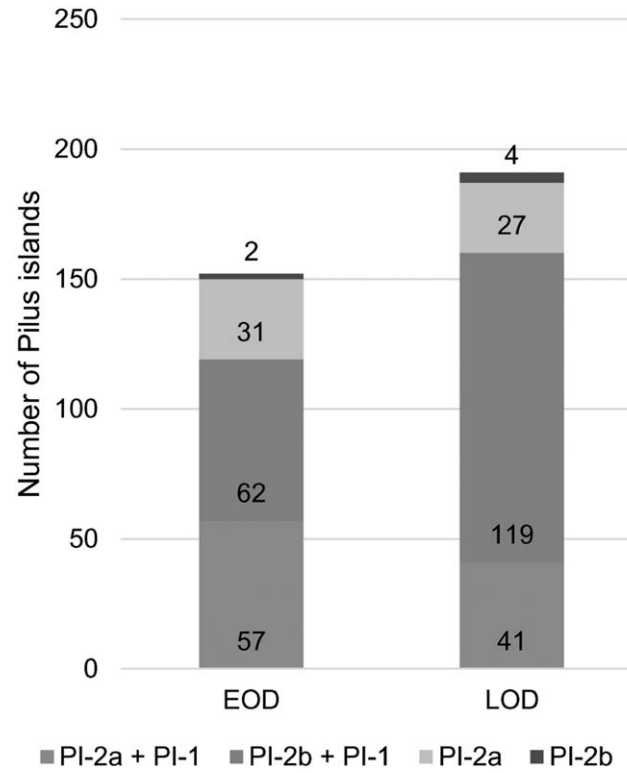

FIGURE 3. Pilus island distribution among EOD and LOD (n = 343).

Clustering Based on Virulence Factors

While analyzing genes for serotypes, pilus proteins and alpha-like proteins, it became apparent that certain combinations of virulence factors occurred significantly more often than others. Of all isolates, 86% could be grouped into 5 clusters (Table 3). Cluster 1 showed a significant association with LOD and meningitis, whereas cluster 2 was significantly associated with EOD and the absence of meningitis. Cluster 3, harboring capsule type III and *alp rib*, was not significantly associated with either LOD or meningitis. To verify that these clusters truly represent strains with a very high genetic conformity, an additional analysis of 34 gene loci was conducted. This revealed a clear allelic conformity, with at least 95% and 100% for the majority of gene loci carrying the same allele.

Logistic Regression Analysis

A multivariate logistic regression was calculated to identify the virulence factor with the strongest association with specific clinical presentations (Table 4). The virulence factors of clusters 1, PI-2b + PI-1 had the strongest association with LOD, whereas capsular genotype III was strongly associated with meningitis. Within cluster 2, *alp 5* showed the strongest association with EOD and the absence of meningitis.

DISCUSSION

The microarray represents an efficient, reliable and rapid tool for typing large numbers of GBS with a wide genomic range. It provides clear advantages over established methods such as PFGE or MLST. Microarray and MLST are alternative techniques, whereby the results of typing, unfortunately, are not directly comparable.[25] Nitschke et al[25] defined a hybridization pattern by 11

TABLE 3. Cluster of GBS According to Their Serotypes, Pilus Islands and Alpha-like Proteins (86% of All Isolates with Data to Serotype, Pilus Island and Alpha-like Protein (n = 452) Could Be Assigned to a Cluster)

	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
Serotype	III	Ia	III	V	Ib
Pilus island	PI-2b + PI-1	PI-2a	PI-2a + PI-1	PI-2a + PI-1	PI-2a + PI-1
Alpha-like protein	alp rib	alp 5	alp rib	alp 3	alp bca
Significant association ($P < 0.05$)	LOD Meningitis	EOD No meningitis	None	None	None
Total numbers	220	72	45	28	23

TABLE 4. Multivariate Logistic Regression Analysis to Predict Clinical Presentations

Predictor variables	Dependent Variables	Regression Coefficient B	Significance Level (*P*-Value)	Odds Ratio	CI
PI-2a	EOD	0.677	0.456	1.969	0.332–11.689
Alp 5		2.131	0.009	8.420	1.718–41.260
Serotype Ia		–2.073	0.051	0.126	0.016–1.009
PI-2b + PI-1	LOD	0.596	0.065	1.815	0.963–3.421
Alp rib		0.230	0.611	0.794	0.327–1.928
Serotype III		0.663	0.160	1.940	0.770–4.886
PI-2a	No meningitis	0.056	0.951	1.058	0.173–6.477
Alp 5		1.489	0.051	4.431	0.994–19.758
Serotype Ia		–0.321	0.725	0.726	0.122–4.323
PI-2b + PI-1	Meningitis	–0.107	0.770	0.898	0.438–1.843
Alp rib		–0.448	0.382	0.639	0.234–1.744
Serotype III		1.396	0.014	4.041	1.328–12.295

Predictor variables were the virulence factors of one cluster (cluster 1: PI-2b + PI-1, *alp rib,* serotype III or cluster 2: PI-2a, *alp 5*, serotype 5). "Enter" method was used.

markers of the microarray. This pattern showed a high correlation to STs and clonal complexes. A straight and precise transfer of STs to hybridization patterns or vice versa was not possible. To establish the microarray for GBS as a standard typing method, it would be preferable to include additional probes, for example, probes for improved comparison with MLST or for multi-genetic processes, such as the erythromycin resistance.

Nitschke et al[25] validated the microarray with reference strains and MLST. Additionally, this study's overall accordance rate among the microarray, the latex agglutination test and the erythromycin resistance tests was very high. There were only a few limitations. The latex agglutination test could not determine the serotype in 2.5% of all isolates. Although our study did not conduct PCR testing to determine whether serotype genes were present while proteins were not expressed, others previously have done so.[26] Regarding erythromycin resistance, testing for the important resistance gene *mefA* was not included in the microarray.[24] The discrepancy between 22 isolates being genetically sensitive while also phenotypically resistant may be explained by the putative existence of *mefA* in these isolates. Finally, our study's comparison presents genetic and phenotypic data, which as such, are not fully congruent. Analyses of gene expression were not performed.

The present study is the first one describing 3 different groups of invasive neonatal isolate virulence factors collected over 2 different time periods in a large, nationwide isolates collection. As previously has been described in studies of neonatal invasive GBS infection,[12,16,18,19,23,24] the overwhelming proportion of the genes for serotype III, PI-2b + PI-1 and *alp rib* is clearly evident. Additionally, we were able to show that there was no change in virulence factor distribution, either over time or by geographic region in Germany.

In this study, we were able to evaluate a combination of patients' characteristics and virulence factors. A large number of known associations were confirmed, for example the association of capsular genotype III, *alp rib* and PI-2b + PI-1 with LOD and meningitis. Additionally, new associations were able to be shown, such as that of *alp 5* and EOD, along with the absence of meningitis. To date, most studies have evaluated the association between one virulence factor and one clinical characteristic. But with the microarray, we were able to place different virulence factors into a single context. Using multivariate logistic regression, we were able to demonstrate that a given virulence factor had the strongest association to certain clinical characteristics, namely PI-2b + PI-1 to LOD, capsular genotype III to meningitis and *alp 5* to EOD and the absence of meningitis.

Typing GBS with the microarray allows to quickly assign each isolate to a putative strain or cluster. First, this provides a more realistic picture, especially when looking at the associations with clinical data. For example, cluster 3 showed no significant association with LOD or meningitis, despite the fact that capsular genotype III and *alp rib* were present. Second, the microarray technique may be used in routine clinical care to better understand transmission routes or to more quickly distinguish between colonizing and virulent strains.[8]

In summary, our nationwide study covering two, 2-year periods evaluated a quick and practicable new typing method for the encoding genes for capsular genotypes, alpha-like proteins and pilus proteins in GBS isolates. Using the microarray, we were able to show that large epidemiologic studies can be supplemented by genetic analysis. By doing so, we were able to define genetic clusters that strongly were correlated with clinical phenotypes. Additionally, we suggest it may be feasible to use the microarray for diagnostic purposes to provide time-critical information regarding the presence of multiple virulence factors in an individual case, or to elucidate modes of transmission in a given clinical situation or an outbreak.

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