Phylogenetic Lineages of Invasive and Colonizing Strains of Serotype III Group B Streptococci from Neonates: a Multicenter Prospective Study

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This study compares the phylogenetic lineages of invasive serotype III group B streptococci (GBS) to those of colonizing strains in order to determine lineages associated with invasive disease. Isolates from 29 infants with early-onset disease (EOD) and from 196 colonized infants, collected in a prospective, multicenter study, were assigned a sequence type (ST) by multilocus sequence typing. Overall, 54.5% of the isolates were in the ET-19 complex, and 40.4% were in the ST-17 complex. Invasive strains were more likely to be in the ST-17 complex than were colonizing strains (59% versus 38%, P = 0.03). After we adjusted for potential confounders, the ST-17 complex was more likely to be associated with EOD than were other lineages (odds ratio = 2.51, 95% confidence interval = 1.02 to 6.20). These data support the hypothesis that ST-17 complex GBS are more virulent than other serotype III GBS.

Streptococcus agalactiae (group B streptococci [GBS]) is the most common cause of invasive bacterial disease in neonates in developed countries. GBS are classified into nine serotypes according to the immunologic reactivity of the polysaccharide capsule. Serotype III GBS accounts for about 30% of early-onset disease (EOD) (within the first week of life), most late-onset disease (after the first week of life), and the majority of GBS meningitis cases in infants (2, 19).

The clonal structure of the GBS population has been demonstrated by a variety of techniques, including multilocus enzyme electrophoresis, restriction endonuclease digest patterns (RDP) of chromosomal DNA, pulsed-field gel electrophoresis and gene analysis, and, most recently, by multilocus sequence typing (MLST) (8–10, 14–16, 18, 20, 21). These studies have demonstrated that serotype III GBS associated with human disease derive largely from two distinct phylogenetic lineages. Although these two lineages can be identified by any of these techniques, MLST has the advantage of reproducibility and has been shown to correlate with the other techniques and thus has emerged as the standard for delineating the clonal population of GBS (10).

Musser et al. were the first to propose that one lineage of serotype III GBS, called ET-I, is hypervirulent based on its frequent association with invasive disease in human neonates (14). Subsequent studies analyzing RDPs of chromosomal DNA of GBS isolates from Utah and Japan suggest that the RDP type III-3 is a hypervirulent lineage because 91% of the invasive serotype III GBS isolates versus 33% of the colonizing isolates belong to this subtype (20). RDP type III-3 strains and ET-I strains were subsequently shown to be in the same ST-17 clonal complex identified by MLST. The other major serotype III GBS lineage, ST-19 clonal complex, has been shown to be the same as the RDP type III-2 lineage (7, 10, 21).

Other studies, however, have found that the distribution of these two predominant phylogenetic lineages among colonizing isolates was similar to that among isolates from neonates with invasive serotype III GBS disease. A study in Denmark showed that 59% of invasive serotype III GBS isolates and a similar percentage of colonizing isolates were in division V (i.e., ST-17 complex) (8). Most recently, studies of serotype III GBS isolates from Alberta, Canada, showed that the distribution of ST-17 and ST-19 in invasive isolates from neonates (32.1 and 57.1%, respectively) was similar to that of colonizing isolates (5).

These recent reports do not support the earlier observations that suggest the ST-17 lineage is hypervirulent. Most of these observations, however, were based on studies with limitations in their design that precluded an accurate delineation of an association of a specific lineage with invasive disease. Limita-
tions include the absence of an adequate comparison group, failure to take into account maternal and infant risk factors, and the protective effects of immunoglobulin G (IgG) GBS type-specific antibodies.

We have prospectively collected and serotyped both invasive and colonizing isolates of GBS from neonates across multiple centers in the United States (11, 12). We have also collected clinical and epidemiological data from these neonates and their mothers and measured the levels of maternal and cord serum IgG anti-serotype III GBS. In the present study, we performed MLST on invasive and colonizing isolates of serotype III GBS and evaluated whether serotype III GBS ST-17 complex is associated with GBS EOD in neonates by comparing the phylogenetic lineages of invasive isolates to those of colonizing isolates, taking into account risk factors associated with EODs.

**MATERIALS AND METHODS**

**Study population.** Neonates from whom the GBS isolates, serum samples, and clinical and epidemiological data were obtained have been described previously (11, 12). Briefly, we conducted seroepidemiological studies of EOD caused by GBS in six academic centers in Alabama, California, Florida, New York City, New Jersey, and Texas from July 1995 to June 1999. 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. Colonized infants were identified by obtaining positive cultures from the throat, anus, umbilicus, and ears at birth, before the first bath, from a sample of newborns each month. A total of 132 EOD cases were identified; 29 infants were infected by serotype III GBS, including one in whom both serotypes Ia and III GBS were isolated from the blood. Among 1,654 infants colonized with GBS, 330 had serotype III GBS isolated from surface cultures: 97 (29%) were heavily colonized (positive at three or four anatomic sites), and 233 (71%) were lightly colonized (positive at one or two anatomic sites). The study was approved by the Institutional Review Board of each study center.

**Bacterial isolates.** Bacterial isolates from infants with EOD caused by serotype III GBS (cases) were compared to colonizing isolates obtained from healthy infants (controls). For each case, up to four heavily colonized and four lightly colonized infants who were born at the same study center in the same year of the case patient were selected as controls. One isolate from each infant was included in the analysis. In all, 29 invasive isolates, 116 isolates from lightly colonized infants, and 80 isolates from heavily colonized infants who met the control selection criteria were included in this study.

Bacterial isolates, frozen in Todd-Hewitt broth, were sent to the University of Utah, where the isolates were thawed and an inoculum was streaked onto the 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.** MLST was carried out as described previously (10). Each isolate was assigned a sequence type (ST). Isolates were assigned to one of four previously described ST complexes if its ST differed by <3 alleles from the predominant ST in the clonal complex (ST-1, ST-17, ST-19, and ST-23 complex, respectively). The sequences of all novel alleles and the composition of novel STs identified in this study are available at http://sagaactiae.mlst.net.

**Maternal and cord serum IgG anti-serotype III GBS.** Maternal and cord sera were assayed by enzyme-linked immunosorbent assay for IgG anti-serotype III GBS as described previously (12).

**Statistical analyses.** Demographic and obstetric variables were compared among cases and colonized controls. A two-sided chi-square test was used to test for associations between case status and each maternal and infant variable. Classifications of lineages were compared by case status and also by study site. Potential confounders were taken as any demographic or obstetric variable with a P value of ≤0.10 for association with case status. The association of an ST clonal complex with EOD was then assessed by multiple logistic regression, starting with all potential confounders and using backward elimination until all factors had a P value of ≤0.10 for inclusion given all other factors. A cord serum anti-serotype III GBS IgG concentration of less than 2.0 mg/ml was entered as a factor because it has been previously shown to be insufficient to protect neonates from invasive disease (12). Gestational age was entered as a continuous variable and, along with the receipt of antibiotics during labor, was forced to be retained in the final model since they are major factors associated with EOD.

**RESULTS**

**Characteristics of cases and controls.** The 29 cases (19 females and 10 males) were from California (9 cases), Texas (9 cases), Alabama (6 cases), Florida (3 cases), and New York (2 cases). No invasive disease due to serotype III GBS was detected at the New Jersey study site. Cases and controls were similar across a broad range of demographic and obstetric variables, including maternal age, race or ethnic origin, type of insurance, maternal parity and gravidity, prenatal care, maternal prenatal medical conditions, and length of rupture of membranes. There was, however, maternal fever among more cases than controls (24% versus 7%, P = 0.002). Cesarean delivery was also more frequent among mothers of cases than mothers of controls (31% versus 13%, P = 0.01). Seventy-nine percent of the cesarean deliveries were due to fetal distress; 89% among cases versus 75% among controls (P = 0.40).

**Compositions of STs within the clonal complexes.** Of the 225 (29 invasive and 196 colonizing) isolates, 123 (54.7%) were assigned to the ST-19 complex, 91 (40.4%) to the ST-17 complex, 3 (1.3%) to the ST-23 complex, 2 (0.9%) to the ST-1 complex, and 6 (2.7%) to five other lineages. The 123 isolates assigned to ST-19 complex included 10 STs (Table 1). In contrast to previous studies, in which almost all isolates in the ST-19 complex were ST-19, 84% of isolates in ST-19 complex in this study were ST-19. Five (4%) isolates were classified as ST-175 and fifteen isolates as eight other STs (Table 1). The 91 GBS isolates classified as ST-17 complex included 12 STs (Table 2). In contrast to previous reports, in which almost all isolates in ST-17 complex were ST-17, 66% of isolates in ST-17 complex in this study were ST-17. Five (4%) isolates were classified as ST-175 and fifteen isolates as eight other STs (Table 1).

**TABLE 1. Composition of STs in ST-19 complex of serotype III GBS isolated from neonates**

<table>
<thead>
<tr>
<th>ST</th>
<th>Total no. (%) of strains</th>
<th>Invasive strains&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Colonizing strains&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>103 (84)</td>
<td>9 (82)</td>
<td>94 (84)</td>
</tr>
<tr>
<td>175</td>
<td>5 (4)</td>
<td>1 (9)</td>
<td>4 (4)</td>
</tr>
<tr>
<td>193</td>
<td>1 (1)</td>
<td>1 (9)</td>
<td>0</td>
</tr>
<tr>
<td>Other&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14 (11)</td>
<td>0 (0)</td>
<td>14 (13)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Invasive strains were serotype III GBS isolates from blood or cerebrospinal fluid.

<sup>b</sup> Colonizing strains were serotype III GBS isolates from neonate surface swabs.

<sup>c</sup> Seven other STs were identified in the ST-19 complex in this sample.

The 123 isolates assigned to ST-19 complex included 10 STs (Table 1). In contrast to previous studies, in which almost all isolates in the ST-19 complex were ST-19, 84% of isolates in ST-19 complex in this study were ST-19. Five (4%) isolates were classified as ST-175 and fifteen isolates as eight other STs (Table 1). The 91 GBS isolates classified as ST-17 complex included 12 STs (Table 2). In contrast to previous reports, in which almost all isolates in ST-17 complex were ST-17, 66% of isolates in ST-17 complex in this study were ST-17. The remaining were ST-31 (10 isolates) and ST-180 (10 isolates), respectively; 11 additional isolates belonged to nine other STs. The distribution of the STs among the ST-17 complex of invasive strains did not differ significantly from that of colonizing strains, although there was a slightly higher proportion of ST-180 isolates among the invasive than among the colonizing isolates (24% versus 8%, P = 0.087). Isolates from the ST-17 complex with STs other than ST-17 were widely distributed...
TABLE 2. Composition of STs of ST-17 complex of serotype III GBS isolated from neonates

<table>
<thead>
<tr>
<th>ST</th>
<th>Total no. (%) of strains</th>
<th>No. (%) of:</th>
<th>Invasive strainsa</th>
<th>Colonizing strainsb</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>60 (66)</td>
<td></td>
<td>9 (53)</td>
<td>51 (69)</td>
</tr>
<tr>
<td>31</td>
<td>10 (11)</td>
<td></td>
<td>2 (12)</td>
<td>8 (11)</td>
</tr>
<tr>
<td>180</td>
<td>10 (11)</td>
<td></td>
<td>4 (24)</td>
<td>6 (8)</td>
</tr>
<tr>
<td>174</td>
<td>1 (1)</td>
<td></td>
<td>1 (6)</td>
<td>0</td>
</tr>
<tr>
<td>188</td>
<td>1 (1)</td>
<td></td>
<td>1 (6)</td>
<td>0</td>
</tr>
<tr>
<td>Otherc</td>
<td>9 (10)</td>
<td></td>
<td>0 (0)</td>
<td>9 (12)</td>
</tr>
<tr>
<td>Total</td>
<td>91 (100)</td>
<td></td>
<td>17 (100)</td>
<td>74 (100)</td>
</tr>
</tbody>
</table>

a Invasive strains were serotype III GBS isolates from blood or cerebrospinal fluid.
b Colonizing strains were serotype III GBS isolates from neonate surface swabs.
c Seven other STs were identified in the ST-19 complex in this sample.

The frequencies of preterm birth, Apgar score of ≤6 at 1 min, meningitis, or discharge to home in cases caused by ST-17 complex were similar to frequencies for cases caused by ST-19 complex.

Geographic distribution of lineages. The frequency of isolates classified to the ST-17 complex ranged from 21.2% in California to 60.0% in Florida, while the frequency of isolates classified to the ST-19 complex ranged from 30.8% in New York to 74.7% in California (Table 4), a distribution that differed significantly among study sites (P < 0.001, chi-square test). Isolates from California were more likely to belong to the ST-19 complex than isolates from other sites (74.7% versus 45.5%, P < 0.001). Conversely, isolates from California were less likely to be in the ST-17 complex than were isolates from other sites (21.1% versus 49.4%, P < 0.001).

Maternal serum IgG anti-serotype III GBS by lineage. Maternal serum samples were available for assay from 28 cases and 184 controls. The geometric mean (GM) concentration of IgG anti-serotype III GBS of mothers of neonates infected with ST-19 complex isolates (GM = 4.26 µg/ml; range, 0.51 to 104.33, 95% confidence interval [95% CI] = 3.54 to 5.13) was similar to that of mothers of neonates infected with ST-17 complex isolates (GM = 3.77 µg/ml; range, 0.58 to 27.04, 95% CI = 3.15 to 4.50) (P = 0.35). Cord serum samples from 27 cases and 193 controls were available for assay. The cord serum GM concentrations of IgG anti-serotype III GBS obtained from neonates infected with ST-19 complex GBS and ST-17 complex GBS were not significantly different (3.46 versus 2.80 µg/ml, P = 0.09).

Association between EOD and serotype III GBS ST-17 complex. As noted above, a significantly higher proportion of invasive isolates than of colonizing strains were in the ST-17 complex (58.6% versus 37.8%, P = 0.03). The association between EOD and serotype III GBS lineage was evaluated by multiple logistic regression analyses to adjust for potential confounding maternal or infant risk factors (Table 5). The
odds ratio for early-onset infection with ST-17 complex serotype III GBS was estimated at 2.51 (95% CI = 1.02 to 6.20) (Table 5) after we adjusted for the infant's gestational age, cord serum IgG anti-serotype III GBS concentration, presence of maternal fever, receipt of antibiotics during labor, and cesarean delivery. The odds ratio approximates the relative risk when the incidence is rare. Since the incidence of serotype III GBS is rare (estimated at 0.3/1,000 live births based on an overall incidence of EOD at 1/1,000), our data suggest that ST-17 complex serotype III GBS are ~2.5 times more likely than other serotype III GBS lineages to cause EOD.

DISCUSSION

In this study, we used MLST to identify the phylogenetic lineages in a large sample of epidemiologically well-characterized invasive and colonizing isolates of serotype III GBS. These isolates were collected systematically and prospectively from geographically diverse areas in the United States. MLST has distinct advantages over other molecular typing techniques, most notably its reproducibility within and between laboratories.

There are several interesting observations from these results. First, the proportion of isolates in the ST-17 and ST-19 clonal complex varied among different geographic sites. This observation, along with the variable rates of infection by ST-17 complex GBS reported in previous studies, suggests that the carriage rate for the two lineages among pregnant women is dynamic and subject to unknown influences. Second, the STs in the two lineages, particularly the ST-17 complex, are more diverse than previously described, with some subclones demonstrating a regional distribution, suggesting a recent introduction and/or clonal expansion within a geographic area. Third, the study confirms that almost all invasive neonatal serotype III GBS disease in the United States is associated with just two lineages, which also comprise the major populations that colonize neonates and, by inference, their mothers. Fourth, serotype III GBS from infants with EOD were more likely to be in the ST-17 clonal complex than in other phylogenetic lineages, whereas isolates from infants who were colonized and did not develop disease were more likely to be in the ST-19 clonal complex. In addition, we showed that the clinical outcomes of cases caused by ST-17 complex were similar to cases caused by the ST-19 complex. The GM concentration of maternal IgG GBS type III in mothers of ST-17 complex-infected newborns did not differ significantly from that of mothers of ST-19 complex-infected newborns. We further assessed the association between the ST-17 lineage and EOD by multiple logistical analyses, adjusting for potential confounders, demonstrating that ST-17 GBS were 2.5 times more likely than the GBS of other ST complexes to cause invasive disease.

Heavy colonization with GBS at birth is known to increase the risk of invasive disease over that caused by light colonization (6), but in this study sample, the percentages of serotype III GBS from lightly colonized isolates and heavily colonized isolates in the ST-17 complex were not significantly different. Thus, the increased risk of infection associated with ST-17 complex GBS did not result from a tendency of ST-17 GBS to be associated with heavy colonization.

In an earlier study, we proposed that the basis for the presumed increased virulence of RDP type III-3 (ST-19 complex) GBS was that strains in this complex possessed a higher sialic acid content than RDP type III-2 (ST-19 complex), suggestive of a larger size of capsular polysaccharide of the RDP type III-3 GBS compared to the RDP type III-2 GBS, which resulted in resistance to opsonization by complement (20). The expression of hyaluronate lyase in RDP type III-3 GBS and the lack of expression of hyaluronate lyase in RDP type III-2 GBS could also contribute to the difference between the clinical behaviors of RDP type III-3 and III-2 GBS (21). Other factors may contribute to the different clinical behaviors of the two lineages, which are genetically quite distinct from each other. In fact, ST-17 GBS are more closely related to a clonal complex that causes bovine mastitis than to ST-19 GBS (3, 4).

Studies published by both Hauge et al. and Davies et al. did not identify ST-17 complex GBS as a risk factor for EOD (5, 8). The explanation for the discrepancy between their findings and our own is not clear but could be related to the lack of an adequate control group, to the study of populations with different demographic or clinical features, or to the presence of unique bacterial subpopulations with increased or decreased pathogenic potential. Indeed, we found that invasive ST-17 complex GBS in our study included ST-180 strains, whereas this ST was not identified in the population studied by Davies et al. (5). The results of this study do not allow us to conclude whether ST-180 isolates are more virulent than other strains within the ST-17 complex, nor whether the 14 non-ST-19 colonizing strains in the ST-19 complex were less virulent than the other III-2 strains. Additional analyses, including the analysis of putative virulence factors such as bacterial surface proteins or enzymes with specific lineages (13, 17) or the susceptibility of bacteria from each lineage to IgG-independent host defense mechanisms, such as L-ficolin (1), could help determine what host and bacterial factors lead to the disproportionate ability of ST-17 complex GBS strains to cause invasive disease.

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REFERENCES


