The populations of group B streptococcus (GBS) associated with vaginal carriage in pregnant women and invasive neonatal infections in Portugal were compared. GBS isolates were characterized by serotyping, pulsed-field gel electrophoresis (PFGE) profiling, and multilocus sequence typing (MLST). Serotypes III and V accounted for 44% of all colonization isolates (n = 269), whereas serotypes III and Ia amounted to 69% of all invasive isolates (n = 64). Whereas serotype Ia was associated with early-onset disease (EOD), serotype III was associated with late-onset disease (LOD). Characterization by PFGE and MLST identified very diverse populations in carriage and invasive disease. Serotype Ia was represented mainly by a single PFGE cluster defined by sequence type 23 (ST23) and the infrequent ST24. In contrast, serotype III was found in a large number of PFGE clusters and STs, but a single PFGE cluster defined by ST17 was found to be associated with invasive disease. Although serotype III was associated only with LOD, ST17 showed an enhanced capacity to cause both EOD and LOD. Our data reinforce the evidence for enhanced invasiveness of ST17 and identify a lineage expressing serotype Ia capsule and represented by ST23 and ST24 as having enhanced potential to cause EOD.

Streptococcus agalactiae, or group B streptococcus (GBS), emerged during the 1960s as an important cause of neonatal disease, and by the 1970s, it was already established as a leading cause of infections in the newborn (19, 21, 30). In neonates and infants, GBS disease is defined as either early-onset disease (EOD) (age, 0 to 6 days) or late-onset disease (LOD) (age, 7 to 90 days) (10). EOD is associated with the presence of GBS in the vagina of the mother, and transmission is thought to occur vertically through aspiration of infected amniotic fluid or passage through the birth canal. Several studies have documented the serotypes of isolates colonizing the vaginas of pregnant women and those causing invasive infections in newborns (18, 27, 30, 36). The source of bacterial strains causing LOD is less well understood and may involve community or nosocomial acquisition, although there is also evidence that in some infants with LOD, the GBS causing the infection shares the same serotype as the GBS isolated from their mothers, suggesting a maternal source (30).

Although, prevention of GBS neonatal infections by antimicrobial prophylaxis was suggested as early as the mid 1960s and a selective screen for carriage in pregnant women was also proposed a few years later (19), it was not until 1996 that guidelines for the prevention of GBS neonatal infections were published in the United States (10). The initial guidelines suggested a mixed risk-based and screening-based approach, but later guidelines suggested the universal screening of pregnant women for GBS vaginal colonization at 35 to 37 weeks of gestation and the administration of intrapartum antibiotics to carriers (9). The implementation of these guidelines resulted in a massive decrease in EOD but has not affected the rate of LOD (8). Moreover, it was also noted that antimicrobial prophylaxis could have unwanted long-term effects due to increased antimicrobial use (31), and alternative prevention strategies have focused on the development of vaccines. Vaccine formulations based on the conjugation of GBS capsular polysaccharides to tetanus toxoid have already undergone phase I and II clinical trials, and studies evaluating their potential impact in the management of GBS disease suggest that vaccination may provide additional benefits over antimicrobial prophylaxis, especially due to the expected reduction in LOD (34). As an alternative or complement to these conjugate vaccines, efforts have been directed toward identifying bacterial surface proteins that could be used in vaccination (26).

To supplement these approaches, the genetic lineages responsible for neonatal infections and vaginal colonization were characterized, with the objective of identifying particularly virulent clones. Recent studies have relied on multilocus sequence typing (MLST) and have identified a serotype III lineage defined by sequence type 17 (ST17), of bovine origin, as having enhanced virulence (3, 23). However, these comparative studies have been carried out in only two geographic areas (3, 4), and it would be of interest to perform these studies in other regions, where GBS disease may present different characteristics, to test the global validity of these findings.

We undertook the characterization of GBS isolates recovered from vaginal carriage in pregnant women screened at 35 to 37 weeks of gestation and isolates responsible for invasive infections in infants in Portugal with the aim of identifying particular genetic lineages with enhanced virulence. The over-representation of serotype III, ST17, among neonatal invasive isolates was confirmed, and this lineage was responsible for

---

* Corresponding author. Mailing address: Instituto de Microbiologia, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal. Phone: 351-21 799 9460. Fax: 351-21 799 9459. E-mail: ramirez@fm.ul.pt.
† E.R.M. and M.A.P. contributed equally to this work.
‡ Published ahead of print on 15 August 2007.
The bacteria were isolated during the normal antenatal follow-up of women in pregnancy, using the recommended procedures for enhanced recovery of GBS (9). Rectal swabs of healthy asymptomatic women in their last trimester of pregnancy were obtained. A total of 225 isolates were analyzed by PFGE and MLST. Capsular serotyping was done by slide agglutination using sera for types Ia, Ib, and II to VIII (hemolytic streptococci typing antisera for group B; SEIKEN, Japan) according to the instructions of the manufacturer. Preparation of genomic DNA for pulsed-field gel electrophoresis (PFGE) analysis was done as described elsewhere (16). After digestion with SmaI (Fermentas, Vilnius, Lithuania), the fragments were resolved by PFGE as previously described (16). Comparison of PFGE patterns was performed by using Bionumerics software (Applied-Maths, Sint-Martens-Latem, Belgium) to create unweighted-pair group method with arithmetic means dendrograms. The characterization by MLST of selected isolates was performed as described previously (16). Comparison of PFGE patterns was performed by using Bionumerics software (Applied-Maths, Sint-Martens-Latem, Belgium) to create unweighted-pair group method with arithmetic means dendrograms. The Dice similarity coefficient was used with optimization and position tolerance settings of 3.0 and 1.5, respectively. Clones were defined as groups of isolates (n ≥ 3) presenting profiles ≥80% related on the dendrogram, as previously described for Streptococcus pneumoniae (33). The choice of this cutoff value for the definition of clones is supported by prior work on other streptococcal species showing that this value minimized incorrect classifications due to the inherent variability of the PFGE analysis (6) and that the groups defined showed extensive concordance with those defined by visual classification systems (33), as well as with those defined using other typing methods (6, 7).

TABLE 1. Enhanced disease potentials of S. agalactiae serotypes

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Total no. of isolates</th>
<th>No. of colonization isolates</th>
<th>No. of infection isolates</th>
<th>OR (95% CI)</th>
<th>No. of EOD isolates</th>
<th>OR (95% CI)</th>
<th>No. of LOD isolates</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia</td>
<td>60</td>
<td>42</td>
<td>18</td>
<td>2.11 (1.12–4.00)</td>
<td>13</td>
<td>2.42 (1.16–5.04)</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>Ib</td>
<td>16</td>
<td>14</td>
<td>2</td>
<td>NS</td>
<td>1</td>
<td>NS</td>
<td>1</td>
<td>NS</td>
</tr>
<tr>
<td>II</td>
<td>54</td>
<td>46</td>
<td>8</td>
<td>NS</td>
<td>8</td>
<td>NS</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>III</td>
<td>85</td>
<td>59</td>
<td>26</td>
<td>2.44 (1.37–4.34)</td>
<td>12</td>
<td>14</td>
<td>6.23 (2.49–15.56)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>8</td>
<td>6</td>
<td>2</td>
<td>NS</td>
<td>1</td>
<td>NS</td>
<td>1</td>
<td>NS</td>
</tr>
<tr>
<td>V</td>
<td>66</td>
<td>59</td>
<td>7</td>
<td>NS</td>
<td>7</td>
<td>NS</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>VII</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>NA</td>
<td>0</td>
<td>NA</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>NTb</td>
<td>39</td>
<td>38</td>
<td>1</td>
<td>0.10 (0.01–0.72)</td>
<td>0</td>
<td>NA</td>
<td>1</td>
<td>NS</td>
</tr>
<tr>
<td>Total</td>
<td>333</td>
<td>269</td>
<td>64</td>
<td>42</td>
<td>22</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a An OR of >1 indicates increased invasive-disease potential. NS, not significant; NA, not applicable.

RESULTS

Capsular serotyping. The results of serotyping the 64 invasive GBS isolates from infants and the 269 isolates from asymptomatic colonization of pregnant women recovered in the last trimester of pregnancy are summarized in Table 1. Serotypes III and V were the most prevalent among asymptomatic-colonization isolates, together accounting for 44% of all colonization isolates, whereas serotypes Ia and III were the most prevalent among invasive-disease isolates, together accounting for 69% of the isolates. There were more isolates recovered from the CSF in cases of LOD (n = 6) than in EOD (n = 3), but the difference was not significant (Fisher’s exact test, P = 0.051). Serotype III was found to be more frequently isolated from CSF than expected from its representation in invasive isolates (Fig. 1), with seven isolates recovered from CSF (Fisher’s exact test, P = 0.025).

PFGE and MLST. A total of 225 isolates were analyzed by PFGE, including all isolates expressing capsular types III (n = 85) and Ia (n = 60); all infection isolates from the remaining serotypes and three randomly chosen colonization isolates for each invasive-infection isolate expressed these serotypes. The clones defined by PFGE are represented in Fig. 1. To further characterize the genetic lineages associated with each PFGE clone, selected isolates (n = 75) were characterized by MLST. Six novel alleles (adhP58, pheS25 and -26, atr37, glkA36, and glkK30) and seven novel STs (ST286, ST288 to ST291, ST293, and ST295) were identified among the studied isolates. Each PFGE cluster was composed mostly of isolates of the same serotype—Wallace index, 0.865, meaning that only 1 out of every 10 pairs of isolates grouped together by PFGE will not share the same serotype—but each serotype could be clearly estimated.
separated into several PFGE clusters (Wallace index, 0.411). However, one of the largest PFGE clusters (cluster C) (Fig. 1) was composed of significant numbers of both serotype III (n = 20) and serotype II (n = 8) isolates. Although these represented STs that were single-locus variants of each other, no ST was found associated with both serotypes. The isolates grouped in each PFGE cluster belonged to the same genetic lineage, as determined by MLST and eBURST analysis, being single-locus variants of double-locus variants of at least another isolate in the same PFGE cluster, confirming the usefulness of PFGE for identifying GBS clones.

Estimation of the invasiveness of serotypes and PFGE-defined clones. The OR were calculated for all serotypes identified among invasive isolates, and the results are presented in Table 1. The distribution of the capsular serotypes between EOD and LOD was not homogeneous. To test if the enhanced invasiveness of some serotypes was correlated with an association with each of the disease manifestations, the OR were also calculated by comparing the isolates recovered from vaginal colonization and EOD and LOD separately, and the results are also summarized in Table 1. The two serotypes with higher OR for disease (Ia and III) were found to have solely higher and significant OR for EOD and LOD, respectively. The majority of isolates of serotype Ia were clustered together in the same PFGE group (cluster A) but presented three distinct STs (ST23, ST24, and ST144); however, they are at most double-locus variants of each other and belong to the same eBURST group (Fig. 1). In contrast, isolates presenting serotype III were found in various PFGE clusters, the two larger ones clearly distinguishing the ST17 lineage (cluster B) and the ST19 lineage (cluster C). The majority of the serotype III isolates responsible for invasive disease were found in the ST17 lineage (n = 19/26). Isolates causing infection belonged more frequently to this lineage than to any other lineage found within serotype III (Fisher’s exact test, P = 0.0003). To further explore the invasive potential of this lineage, we calculated the OR for the PFGE cluster exhibiting ST17 against all other isolates, assuming that none of the isolates expressing serotypes other than III would present this ST. We believe this to be a reasonable assumption, since no such isolates have been described in the literature to date. An enhanced invasive-disease potential was found for ST17 for both EOD (OR = 4.63; 95% CI, 1.95 to 10.98) and LOD (OR = 10.59; 95% CI, 4.10 to 27.34). A similar approach for the PFGE cluster with isolates presenting ST23 and ST24 showed an enhanced EOD potential (OR = 2.23; 95% CI, 1.08 to 4.62), but not for LOD (OR = 2.05; 95% CI, 0.80 to 5.23).

DISCUSSION

Similar to previous studies, we found a diverse population among GBS colonization isolates, as well as among those causing invasive disease in Portugal, not only in terms of capsular polysaccharides, but also in genetic lineages defined by both PFGE and MLST (Table 1 and Fig. 1). Apart from serotypes VI and VIII, all other serotypes were found among our collection, and all except serotype VII were associated with both carriage and infection. Among the isolates causing infection, two isolates were identified expressing serotype IV, a serotype frequently associated with carriage in Asia (1) but infrequently found as a cause of neonatal infections in Western countries (29). Also noteworthy was the high prevalence of ST24 found exclusively among isolates of PFGE cluster A (n = 7/18 isolates characterized by MLST). ST24 was described in the publication proposing the GBS MLST scheme in a single isolate of serotype Ia (22) but has rarely been found among large collections of GBS isolates characterized by MLST since then; for instance, only 1.9% of 369 isolates, including both colonization and infection isolates, recovered in the Oxford region presented ST24 (23). None of the PFGE clones with ≥5 isolates could be solely associated with colonization or infection (Fig. 1), indicating that all major lineages are capable of both asymptomatic colonization and causing invasive disease. The isolates belonging to each serotype were dispersed in a widely variable number of PFGE clusters, from only 2 in serotype Ia to 19 in serotype III isolates.

When the population associated with carriage and the one causing infection were compared, serotypes Ia and III were found to have increased invasive-disease potential. On the other hand, nontypeable isolates, frequently representing variants that express little or no capsular polysaccharide, which is an important GBS virulence factor (32), showed a lowered invasive-disease potential. However, there was a clear asymmetry in the prevalence of the various serotypes in EOD and LOD, and a more detailed analysis, stratified by early and late-onset infections, indicated that serotype Ia had a significant OR for EOD while serotype III was significant only in LOD. Serotype III was also overrepresented in isolates recovered from the CSF, in agreement with previous studies suggesting an association of this serotype with meningitis (20). A higher proportion of LOD caused by serotype III is not unusual, and several reports, both from Europe (2, 13, 37) and from the United States and Canada (11, 20), have documented the prominent roles of serotypes Ia and III in EOD and LOD, respectively. None of these reports, however, offered data regarding serotype prevalence in vaginal colonization, preventing...
an evaluation of the invasive potentials of these serotypes in these contexts.

Among serotype III isolates, two main lineages were distinguished by PFGE and MLST—a PFGE cluster represented exclusively by ST17 and a PFGE cluster represented by ST19 and associated STs, with the former being significantly associated with infection. These findings are in agreement with previous suggestions that the ST17 lineage constitutes a particularly virulent lineage (4, 22, 23) and that ST19 is mostly associated with carriage (25). The two studies that suggested an enhanced virulence potential for the ST17 lineage did not distinguish between EOD and LOD (4, 22), but a later study in the Oxfordshire, United Kingdom, region found a significant association of the ST17 lineage with both EOD and LOD (23).

When calculating OR, the implicit assumption is that one is comparing the distribution of serotypes in the reservoir to that of the one causing disease. A higher representation of a particular serotype or clone among the isolates causing disease can then be interpreted as a higher disease potential of that particular serotype or clone. In the case of neonatal GBS infections, the reservoir is assumed to be the asymptomatic vaginal colonization of pregnant women. Multiple lines of evidence support this assumption for EOD, including the dramatic reduction in EOD brought about by intrapartum antibiotic prophylaxis (8); however, the case for LOD is not so well established. A maternal source was clearly implicated in some cases of LOD, but this was associated with ingestion of contaminated breast milk and not with vaginal colonization (17). Nosocomial acquisition of GBS was shown to occur (12, 28) and to be a possible cause of LOD (24), but its prevalence remains unknown, as well as the ultimate source of these isolates. Colonization of the human host is not restricted to the vagina and gastrointestinal tract but was also shown to occur in the upper respiratory tract, which could also be an important reservoir for GBS (15) and a significant source for transmission of these bacteria to infants. These data argue for caution when interpreting OR calculated by including isolates causing LOD.

Since serotype III showed only a significantly enhanced potential to cause LOD but was also a serotype including a large number of distinct clones, we calculated the OR for the PFGE cluster exhibiting ST17 against all other isolates. An enhanced invasive-disease potential of ST17 for both EOD and LOD was found, confirming a previous report (23) but in contrast to the results obtained when all serotype III isolates were considered (Table 1). A similar approach for the PFGE cluster with isolates presenting ST23 and ST24 showed an enhanced EOD potential, but not for LOD, in line with the results for serotype Ia, as expected from the genetically homogeneous nature of this serotype in our collection.

The characteristics of GBS associated with carriage and responsible for invasive neonatal infections in Portugal were similar to those of comparable populations from different geographic areas. Our data identified the genetically homogeneous serotype Ia as having enhanced potential to cause EOD and confirmed the identification of the ST17 lineage, expressing serotype III, as having enhanced potential to cause both EOD and LOD, although the interpretation of the values for LOD warrants caution due to the uncertain nature of the reservoir for these infections. Most prior studies did not distinguish between EOD and LOD for the calculation of OR, and this may have prevented the identification of the enhanced potential of serotype Ia clones to cause EOD. The unusually high proportion of ST24 isolates among serotype Ia found in our collection may also have influenced the recognition of an enhanced capacity of this serotype to cause EOD, since prior studies did not find ST23 to be particularly virulent (23). Similar to the way in which the case for enhanced invasive-disease potential of ST17 was strengthened by the independent study of geographically separated populations, the propensity of serotype Ia to cause EOD should be evaluated elsewhere.

ACKNOWLEDGMENTS

This work was partly supported by Fundação para a Ciência e Tecnologia (POCI/US-E/C/576/406/2004).

The members of the Portuguese Group for the Study of Streptococcal Infections are as follows: Paulo Lopes, Isamila Calheiros, Luísa Felício, and Lourdes Sobral (Centro Hospitalar de Vila Nova de Gaia, Vila Nova de Gaia, Portugal); Rosa M. Barros, Maria Isabel Peres, and Isabel Daniel (Hospital D. Estefânia, Lisbon, Portugal); José Diogo, Ana Rodrigues, and Isabel Nascimento (Hospital Garcia de Orta, Humaitá, Portugal); Francisco Xavier, Ana Marques, and José Salgado (Hospital de Santa Maria, Lisboa, Portugal); Ana Paula Castro, Maria Helena Ramos, and José M. Amorim (Hospital de Santo António, Porto, Portugal); Filomena Martins and Elsa Gonçalves (Hospital de S. Francisco Xavier, Lisbon, Portugal); Fernando Cotta, Maria José Machado Vaz, and Cidália Pina-Vaz (Hospital de São João, Porto, Portugal); Maria Albert Faustino and Adelaide Alves (Hospital de São Marcos, Braga, Portugal); Ana Paula M. Vieira (Hospital Senhora da Oliveira, Guimarães, Portugal); Ana Paula Castro (Hospital de Vila Real Vila Real, Portugal); and Isabel Lourenço (Maternidade Alfredo da Costa, Lisbon, Portugal).

REFERENCES


