UNIVERSIDADE DE LISBOA
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Streptococcus agalactiae causing human infections: genetic diversity and capsular switching

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DOUTORAMENTO EM CIÊNCIAS E TECNOLOGIAS DA SAÚDE
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SUMMARY

Keywords: *Streptococcus agalactiae*, genetic lineages, capsular switching

Streptococcus agalactiae (group B streptococci, GBS) is primarily a colonizing agent of the genitourinary and gastrointestinal tracts of a significant proportion of the human population. It is, however, well established as a leading cause of bacterial sepsis and meningitis in neonates and is increasingly associated with invasive infections in adults.

While vertical transmission is commonly accepted to be the cause of early-onset disease, the source of bacterial strains causing infection in the late-onset period is less well understood. Administration of intrapartum antimicrobial prophylaxis to colonized women has resulted in a striking decline in early-onset and maternal GBS disease, but late-onset infections have mostly remained unchanged. Moreover, antimicrobial prophylaxis raised concerns as to selection and emergence of GBS resistant strains and alternative prevention strategies have focused on the development of vaccines that hold promising, although still preliminary results.

The aim of the work presented in this thesis was to characterize the population structure of GBS in Portugal, and to assess the genetic diversity of isolates recovered from vaginal colonization and invasive disease in different age groups, to contribute to the global epidemiology of GBS and our understanding of GBS population biology. To this end a set of common techniques was chosen, including serotyping, antimicrobial susceptibility testing, pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and surface protein gene profiling. In combination, these methods allowed the identification of the main genetic lineages circulating in Portugal and Barcelona, providing the means for an appropriate comparison of both.

These studies started with the comparison of 64 isolates recovered from invasive infections in newborns in the Lisbon area with 269 isolates colonizing women in the third trimester of pregnancy, from the same period. The genetic lineages defined by both PFGE and MLST identified very diverse populations with reported differences in the prevalence of serotypes and clones in carriage and invasive disease. A major finding concerned the identification of an unusually high proportion of ST24 isolates among serotype Ia, further strengthened by the independent study of another population (212 neonatal isolates) from the Barcelona area. Despite the geographic distance, both studies

from Barcelona and Lisbon revealed extensive similarity in terms of clonal structure and genetic lineages. The high prevalence in both the studies of a particular lineage serotype Ia, defined by ST24 and the surface protein gene *bca*, highlighted the importance of local dynamics, indicating that genetic evolution of GBS presents with a geographic structure and may depend on local factors.

The subsequent analysis of 225 isolates recovered from non-pregnant adults in Portugal revealed a GBS population dominated by a more diverse clonal composition when compared to that of neonates, consistent with the broader spectrum of disease presentation in these patients and consequent multiplicity of genetic lineages. Invasive disease in this population increased with age and was more frequent among men. The dominance of serotype Ia in this population, regardless of age, highlighted the importance of this serotype in GBS pathogenesis as a leading cause of invasive infections in adults, not reported elsewhere but already noted among neonatal infections in the Iberian Peninsula. Furthermore, the high prevalence of ST24 in all these studies, as opposed to rare descriptions elsewhere, suggested that this lineage had enhanced invasiveness and was probably expanding as a regionally successful clone that may disseminate more globally.

Macrolide resistance rates in Portugal did not show significant trends, even if macrolides have been used in intrapartum prophylaxis increasing the selective pressure on GBS. Macrolide resistance is disseminated in Portugal by both a multiclonal mechanism resulting from the spread of resistance genes throughout most serotypes and genetic backgrounds, as well as by clonal expansion of particular lineages, such as the serotype V ST1/*alp*3.

One of the main purposes of the analysis of a significant number of GBS isolates was their classification into lineages sharing the same genetic background, which would allow the inference of genetic relationships between strains and their contextualization in the global epidemiology of GBS. However, the associations of phenotype-genotype or between different genetic traits were never absolute, highlighting the role of horizontal genetic transfer in the evolution of GBS. Capsular switching was anticipated to occur frequently within GBS, even though this species is not recognized to be naturally competent for the acquisition of foreign DNA. Substantial evidence provided by the epidemiological studies performed on the Portuguese GBS collections drove the search for capsular transformants within these populations. The results obtained confirmed the existence of capsular switching in GBS, but questioned the high

frequency of these events estimated from previous studies. Serotyping errors probably justified the overrepresentation of capsular switching in epidemiological studies. The mechanism for these genetic transfer events involved the replacement of the whole capsular locus instead of the previously proposed genetic transfer of only the serotype-specific genes.

Globally, the results presented in this thesis suggest that GBS has an apparent remarkably stable, both temporally and geographically, clonal structure. Against this, background diversification is ongoing and can depend on local factors. Capsular switching is likely contributing to diversification, however not as frequently as initially thought and may impact on the vaccine formulations currently under development. Despite increasing information on maternal colonization and invasive disease, a better understanding of colonization in adults and natural reservoirs of GBS is required for the appropriate management of the GBS infections.

RESUMO

Palavras-chave: *Streptococcus agalactiae*, linhagens genéticas, transformação capsular

Streptococcus agalactiae (estreptococos do grupo B, GBS) é maioritariamente um agente colonizador dos tratos genito-urinário e gastrointestinal de uma proporção significativa da população humana. É, no entanto, reconhecido como uma das principais causas de sépsis e meningite bacteriana em recém-nascidos, e crescentemente associado a infecções invasivas em adultos.

Enquanto a transmissão vertical é normalmente aceite como causa do aparecimento precoce da doença, a origem das estirpes bacterianas que causam infecção no período tardio é menos bem compreendida. A administração de profilaxia antimicrobiana intraparto a mulheres colonizadas resultou num declínio acentuado das infecções por GBS de início precoce e materna, mas as infecções de início tardio, na sua maioria, mantiveram-se inalteradas. Mais ainda, a profilaxia antimicrobiana levantou preocupações quanto à selecção e ao aparecimento de estirpes de GBS resistentes e as estratégias alternativas de prevenção têm-se focado no desenvolvimento de vacinas com resultados promissores, embora ainda preliminares.

O objectivo do trabalho apresentado nesta tese foi caracterizar a estrutura da população de GBS em Portugal e avaliar a diversidade genética das estirpes isoladas de colonização vaginal e doença invasiva em diferentes faixas etárias, de forma a contribuir para a epidemiologia global de GBS e para a compreensão da biologia populacional de GBS. Para atingir esse objectivo foi escolhido um conjunto de técnicas, incluindo a serotipagem, testes de susceptibilidade a antimicrobianos, electroforese em gel de campo pulsado (PFGE), "multilocus sequence typing" (MLST) e determinação de perfis de proteínas de superfície. Em combinação, estes métodos permitiram a identificação das principais linhagens genéticas que circulam em Portugal e Barcelona, fornecendo meios para uma comparação apropriada de ambas.

Estes estudos começaram com a comparação de 64 estirpes isoladas de infecções invasivas em recém-nascidos na região de Lisboa com 269 estirpes de colonização em mulheres no terceiro trimestre de gravidez, no mesmo período. As linhagens genéticas definidas por PFGE e MLST identificaram populações muito diversificadas, com diferenças descritas na prevalência dos serótipos e clones, em colonização e doença invasiva. Uma constatação importante diz respeito à identificação de um número

excepcionalmente elevado de estirpes ST24 no serótipo Ia, reforçado pelo estudo independente de outra população (212 estirpes neonatais) da área de Barcelona. Apesar da distância geográfica, ambos os estudos de Barcelona e Lisboa revelaram uma enorme semelhança em termos da estrutura clonal e linhagens genéticas. A elevada prevalência em ambos os estudos de uma linhagem particular do serótipo Ia, definida pelo ST24 e pelo gene que codifica a proteína de superfície *bca*, salientaram a importância de dinâmicas locais, indicando que a evolução genética de GBS apresenta uma estrutura geográfica e pode depender de factores locais

A análise subsequente de 225 estirpes isoladas de adultos, exceptuando grávidas, em Portugal revelou uma população de GBS dominada por uma composição clonal mais diversificada quando comparada com a de recém-nascidos, consistente com o espectro mais alargado de manifestações de doença nestes indivíduos e consequente multiplicidade de linhagens genéticas. A doença invasiva nesta população aumentou com a idade, sendo mais frequente entre os homens. A predominância do serótipo Ia nessa população, independentemente da idade, destacou a importância deste serótipo na patogénese da GBS como uma das principais causas de infecções invasivas em adultos, não relatada noutros locais mas já identificada nas infecções neonatais na Península Ibérica. Além disso, a elevada prevalência de ST24 em todos estes estudos, por oposição a raras descrições noutros locais, sugerem que esta linhagem tem maior capacidade invasiva e está provavelmente em expansão como um clone bem-sucedido a nível regional que pode disseminar mais globalmente.

As taxas de resistência aos macrólidos em Portugal não apresentaram tendências significativas, ainda que os macrólidos sejam usados na profilaxia intraparto, aumentando a pressão selectiva sobre GBS. A resistência aos macrólidos está disseminada em Portugal, tanto por um mecanismo multiclonal que resulta na dispersão de genes de resistência na maior parte serótipos e clones, como pela expansão clonal de linhagens específicas, tais como a do serótipo V ST1/*alp*3.

Um dos principais objectivos da análise de um número significativo de estirpes de GBS era a sua classificação em linhagens com o mesmo património genético, o que permitiria a inferência de relações genéticas entre estirpes, e a sua contextualização na epidemiologia global de GBS. Contudo, as associações fenótipo-genótipo ou entre características genéticas diferentes nunca foram absolutas, salientando o papel da transferência genética horizontal na evolução de GBS. Foi proposto que a transformação capsular ocorre frequentemente em GBS, apesar de esta espécie não ser

reconhecida como naturalmente competente para a aquisição de DNA exógeno. Evidências substanciais fornecidas pelos estudos epidemiológicos realizados nas colecções portuguesas de GBS levaram à pesquisa de transformantes capsular nessas populações. Os resultados obtidos confirmaram a existência de transformação capsular em GBS, mas questionaram a elevada frequência destes eventos, estimada em estudos anteriores. Erros de serotipagem provavelmente justificam a elevada representação de transformação capsular em estudos epidemiológicos. O mecanismo na base destes eventos de transferência genética envolveu a substituição de todo o *locus* capsular em vez da transferência genética de apenas os genes específicos do serótipo, proposta anteriormente.

Globalmente, os resultados apresentados nesta tese sugerem que os GBS possuem uma estrutura clonal, extremamente estável, tanto geográfica como temporalmente. Por oposição, a diversificação do património genético é permanente e pode depender de factores locais. A transformação capsular provavelmente contribui para esta diversificação, porém não tão frequentemente quanto inicialmente se pensava e pode ter um impacto na formulação das vacinas actualmente em desenvolvimento. Apesar do aumento da informação sobre a colonização materna e a doença invasiva, uma melhor compreensão da colonização em adultos e dos reservatórios naturais de GBS é necessária para uma abordagem mais fundamentada das infecções por GBS.

THESIS OUTLINE

The purpose of the work presented in this thesis was to obtain insights on different aspects of the molecular epidemiology of *Streptococcus agalactiae* with particular emphasis on invasive infections.

Chapter 1 puts the problems addressed in the thesis into context, by briefly reviewing essential aspects of *S. agalactiae* disease and colonization, as well as of the impact of preventive strategies. The contribution of virulence factors and antimicrobial resistance on GBS epidemiology, the importance of surveillance and characterization of the isolates, as well as the major findings in recent years on the structure of the GBS population are also reviewed in this chapter.

Chapters 2 to 5 address the phenotypic and genotypic characterization of the GBS isolated from colonization in pregnant women and invasive infections in newborns and non-pregnant adults.

Chapters 2 and 3 present a detailed description of the epidemiology of GBS isolates recovered from invasive infections in newborns and colonization in pregnant women in Portugal, and of neonatal invasive infections in Barcelona, Spain, respectively. The small number of isolates recovered from invasive infections in neonates in Portugal was complemented by the study of homologous isolates from Barcelona, Spain, with the aim of evaluating if the GBS clonal structure in stable in the Iberian Peninsula.

Chapters 4 and 5 provide a detailed description of the clonal structure of the GBS populations causing invasive infections in non-pregnant adults in Portugal. Chapter 4 addresses the analysis of two temporally and geographically clustered cases of GBS meningitis in adults, rare events that were thoroughly investigated as to their circumstances in the context of GBS invasive disease in Portugal. Chapter 5 consists in the characterization of the isolates recovered from invasive disease in non-pregnant adults in Portugal, in order to assess the main genetic lineages responsible for these infections, and to compare them to those involved in neonatal invasive infections.

Chapter 6 provides unambiguous evidence for the existence of capsular switching in GBS, by recombinational events that imply the exchange of the entire capsular locus rather than switching restricted to capsule-specific genes, as previously proposed in the literature.

Chapter 7 presents an integrated discussion of the major findings of the thesis, highlighting unsolved questions that could be addressed in the future follow-up of these investigations.

Chapters 2-6 can be read independently. They are reproductions of the following publications:

Chapter 2: Martins, E. R., M. A. Pessanha, M. Ramirez, J. Melo-Cristino, and the Portuguese Group for the Study of Streptococcal Infections. 2007. Analysis of group B streptococcal isolates from infants and pregnant women in Portugal revealing two lineages with enhanced invasiveness. J Clin Microbiol 45: 3224-9.

Chapter 3: Martins, E. R., A. Andreu, P. Correia, T. Juncosa, J. Bosch, M. Ramirez, and J. Melo-Cristino. 2011. Group B streptococci causing neonatal infections in Barcelona are a stable clonal population: 18-year surveillance. J Clin Microbiol 49: 2911-2918.

Chapter 4: Martins, E. R., C. Florindo, F. Martins, I. Aldir, M. J. Borrego, L. Brum, M. Ramirez, and J. Melo-Cristino. 2007. *Streptococcus agalactiae* serotype Ib as an agent of meningitis in two adult nonpregnant women. J Clin Microbiol 45: 3850-2.

Chapter 5: E. R. Martins, J. Melo-Cristino, M. Ramirez, and the Portuguese Group for the Study of Streptococcal Infections. 2011. Dominance of serotype Ia among group B streptococci causing invasive infections in non-pregnant adults in Portugal. (Submitted to Journal of Clinical Microbiology)

Chapter 6: Martins, E. R., J. Melo-Cristino, and M. Ramirez. 2010. Evidence for rare capsular switching in *Streptococcus agalactiae*. J Bacteriol 192: 1361-9.

ABBREVIATIONS

Alp	Alpha-like protein
bp	Base pairs
CDC	Centres for Disease Control and Prevention
CLSI	Clinical Laboratory Standards Institute
CPS	Capsular polysaccharide
DLV	Double-locus variant
DNA	Deoxyribonucleic acid
EOD	Early-onset disease
GBS	Group B streptococci
IAP	Intrapartum antibiotic prophylaxis
Ig	Immunoglobulin
Kbp	Kilobase pairs
LOD	Late-onset disease
LS_A	Lincosamide-streptogramin A (resistance phenotype)
M	Macrolide (resistance phenotype)
Mbp	Megabase pairs
MIC	Minimum inhibitory concentration
MLS_B	Macrolide-lincosamide-streptogramin B (resistance phenotype)
MLST	Multilocus sequence typing
NT	Non-typeable
ORF	Open reading frame
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PI	Pilus-island
QRDR	Quinolone resistance determining region
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SLV	Single-locus variant
ST	Sequence type
TT	Tetanus toxoid

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CHAPTER 1

General Introduction

1. *Streptococcus agalactiae*

Streptococcus agalactiae is a Gram-positive bacterium, historically associated to bovine mastitis and dairy sources under the designation of *Streptococcus mastiditis* (101). It was first identified as group B streptococci (GBS) in 1933, when Rebecca Lancefield published her studies on serological differentiation of streptococci (101). GBS was later proposed as an occasional causative agent of puerperal infections (100), and in 1938 it was recognized as an important human pathogen responsible for multiple infections (63). It was not until the 1970s that GBS was acknowledged as a leading cause of neonatal invasive infections (21) and since the 1990s it has also been increasingly associated with invasive infections in non-pregnant adults (54). Despite its unquestionable importance as a human pathogen, GBS is, however, mainly a colonizing agent of the gastrointestinal and genitourinary tracts of a significant proportion of the human population (156).

1.1 Group B streptococcal disease

Neonatal infections

The onset of GBS infections takes place very early in infancy, usually within the first week of life, in which case it is designated of early-onset disease (EOD). Late-onset disease (LOD) develops between one week and three months of age (156). Maternal carriage is a major risk factor for neonatal GBS disease, which is influenced by the degree of bacterial colonization; women with heavy colonization are more likely to have symptomatically infected infants and heavily colonized infants are more likely to develop invasive disease (106).

Whereas EOD and LOD can differ in clinical presentation, mode of transmission and risk factors for disease, the most frequent clinical presentations of invasive disease in neonates are pneumonia, bacteraemia and meningitis. The majority of EOD cases occur within the first 24 hours after birth (156). The onset of disease is associated with the presence of GBS in the genital tract of the mother, and transmission is thought to occur vertically due to an ascending infection during the course of pregnancy or passage through the birth canal (156). Even though perinatal transmission can occur across intact membranes, both premature and prolonged rupture of membranes increase the

risk of GBS acquisition (157). Aspiration of contaminated amniotic fluid then leads to colonization of the airways of the neonate and is rapidly followed by the development of pneumonia. Breaching of the pulmonary mucosal barrier leads to the entry of GBS into the bloodstream and to the development of severe sepsis in some infants (85, 156).

More than half reported cases of neonatal GBS disease now occur during the late-onset period (25). The pathogenesis of LOD is less well understood, although some cases also suggest a maternal source, probably reflecting acquisition of the microorganism during passage through the birth canal (156). Ingestion of contaminated breast milk has also been proposed as a possible maternal source for LOD (10). Even though nearly 50% of mothers of infants with LOD were found to carry the same GBS serotype as that causing infection in their infants, the source of infection in other infants is unclear (156). Nosocomial and horizontal transmission by hospital and community sources are probably involved in some cases of LOD, but the risk factors are not well understood (71). LOD presents with meningitis and bacteraemia without a focus as predominant clinical syndromes; osteoarticular infections, urinary tract infections, and pneumonia are less frequent (157). Meningitis develops when the entry of bacteria into the bloodstream is followed by the invasion of the cerebrospinal fluid. Severe long-term sequelae such as blindness, deafness and global developmental delay occur in a significant number of neonatal meningitis survivors (50).

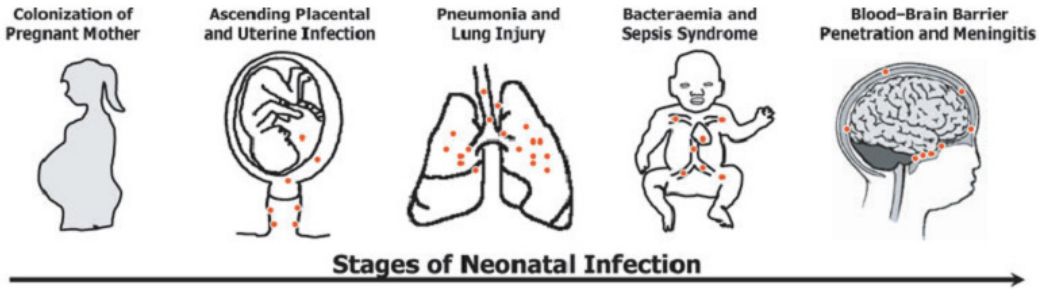

FIGURE 1.1 Stages of neonatal GBS infection. Adapted from (44).

Infections in pregnancy

Pregnant women are at a higher risk of disease because GBS vaginal colonization is a risk factor for maternal peripartum infection (96). The clinical presentations of disease among pregnant women are diverse, and include urinary tract

infection (usually asymptomatic bacteriuria), endometritis, intra-amniotic infection (chorioamnionitis), wound infections associated with caesarean delivery or episiotomy, and, less frequently, puerperal sepsis and meningitis (96, 157). GBS can cause stillbirth in some instances, and evidence points to a causative role of these microorganisms in pre-term delivery (96, 147). An active, laboratory-based surveillance study for GBS infections in the United States revealed that invasive infections among pregnant women accounted for 6.3% of all adult cases of invasive infection (155).

Infections in non-pregnant adults

In the past two decades GBS has been increasingly associated with invasive disease in non-pregnant adults (9, 130, 144, 160). Such infections increase with age, occur more frequently among nursing facility residents than in the community, and are considered responsible for substantial morbidity and mortality, with case-fatality rates of nearly 25% (53, 76). Most cases occur in individuals with significant underlying conditions; diabetes mellitus being is the most frequent co-morbidity, typically present in approximately 30% of non-pregnant adults with GBS disease (54, 82). Other risk factors have been detailed in recent years and include liver cirrhosis, heart and neurologic disease, cancer and immunosuppressive conditions (82).

The clinical spectrum of GBS disease in adults is broad, including more frequently bacteraemia with or without sepsis, skin and soft tissue, osteoarticular and urinary tract infections (53). Less frequent clinical presentations include meningitis and endocarditis that are, however associated with significantly higher morbidity and mortality (43, 152). The possible emergence of GBS as a respiratory pathogen associated with cystic fibrosis has also been proposed in a recent report (52). Recurrent GBS infection was identified among adults, and suggested to be mostly caused by relapse, but an association of relapse with persistent carriage or poor clinical management of primary infection was not determined (74). Nosocomial disease is also raising concerns as more than 20% of patients with GBS invasive infection are thought to have acquired the bacteria from hospital settings (82).

The diversity of clinical presentations and poor outcome of invasive disease in adults are in support for the complexity of the pathogenesis of GBS infections. GBS invasive infections are more frequent in the elderly, probably reflecting the impact of

risk factors that increase with age such as co-morbidities, altered integrity of anatomical barriers and immune senescence (49).

1.2 Colonization and Transmission

The gastrointestinal tract serves as the natural reservoir for GBS and is likely to be the source of vaginal and rectal colonization. Approximately 10-30% of pregnant women are colonized with GBS in the vagina or rectum (147), although the colonization can be transient, chronic or intermittent (72). As maternal GBS carriage in the gastrointestinal and/or genital tracts is a prerequisite for EOD, the different prevalence of maternal GBS colonization could help choose preventive strategies. In most European countries, the prevalence of GBS carriage among pregnant women varies between 6.5 and 36%, with most countries reporting colonization rates of 15-20% (6, 174).

Even though colonization among non-pregnant adults is less well known, vaginal and rectal colonization of healthy young and elderly adults have been reported at levels (20-34%) similar to those observed during pregnancy (13, 51, 123). GBS was also found to be likely transmitted between sex partners during pregnancy (61), yet multiple transmission modes may exist (123). An increasing number of studies also suggest that limited interspecies GBS transmission is likely to occur between humans and their livestock (125, 139, 163), further proposing a framework for GBS as a possible zoonotic infection, which can have significant public health implications (125).

1.3 Prevention strategies - advantages and drawbacks

Although prevention of GBS neonatal infections by intrapartum antibiotic prophylaxis (IAP) was suggested as early as the mid 1960s, and a selective screen for carriage in pregnant women was proposed a few years later (73), it was not until 1996 that guidelines for the prevention of GBS neonatal infections were published in the United States (27). These guidelines identified the candidates for IAP according to either a screening-based or a risk-based strategy (27), and this approach led to a 65% decrease in the incidence of EOD in the United States (155). A later update of the national guidelines in 2002 replaced the recommendation for either of these alternatives

by the recommendation for the universal culture-based screening of all pregnant women for GBS vaginal colonization at 35 to 37 weeks of gestation, and the administration of IAP to carriers (28). As expected, an additional reduction in the incidence of EOD was shown by active population-based surveillance after the guidelines were issued (26, 144).

However, the screening approach for the prevention of GBS neonatal disease has proved challenging. Efforts to reduce missed opportunities of prevention should include the appropriate clinical management of women whose GBS colonization status is unknown, particularly those at risk of preterm delivery; and the identification of factors that contribute for false negative screening results (180, 181).

While in the United States the incidences of EOD and LOD have been closely monitored in the last decades, the same did not happen in Europe, with most countries lacking nationwide or local protocols for surveillance and prevention of neonatal GBS disease. By 1999, only 4 out of 29 European countries had available national guidelines for the prevention of neonatal GBS infections (174). Later, as more countries set up prevention protocols, decreasing incidences of EOD were reported (39). Since 2004 Portugal relies on a protocol for screening and prevention of GBS perinatal disease issued by the neonatology section of the Portuguese Society of Paediatrics (2), due to the lack of guidelines from national health authorities. Subsequently, a study based on voluntary report of GBS neonatal disease in Portugal described a reduction in the incidence of disease of nearly 40%, as well as of the case-fatality rates (135), similarly to what had been observed in other European countries (4, 8, 59, 110, 186).

Despite the fact that universal screening policies and IAP have reduced significantly the high morbidity and mortality rates associated with EOD, while also contributing for the prevention of invasive infections in pregnant women (155), several problems remain regarding the management and prevention of GBS neonatal infections. First, this strategy does not prevent LOD, although more than half of reported cases of neonatal GBS disease now occur during the late-onset period (25), with some countries actually describing increasing incidences of LOD (138). It has been suggested that IAP might delay GBS disease onset, rather than prevent it, resulting in increased rates of LOD (25). Secondly, the widespread use of antibiotics in intrapartum prophylaxis may lead to potentially adverse or unintended effects of GBS prevention efforts, including allergic or anaphylactic reactions to the antimicrobials used in IAP, emergence of GBS

strains resistant to standard therapies, and increasing incidence of neonatal infections caused by pathogens other than GBS (153).

Penicillin is the agent of choice for IAP because of effective transplacental crossing, narrow-spectrum of antimicrobial activity and low cost (6). Although GBS is considered universally susceptible to penicillin, recent studies from Japan (89) and the United States (36) reported reduced penicillin susceptibility in a limited number of both non-invasive and invasive isolates, respectively. These isolates showed increased minimum inhibitory concentrations (MICs) to penicillin, mostly associated to mutations in a penicillin-binding protein (PBP-2x), yet the clinical significance of these findings is unknown.

Additionally, the emerging frequency of GBS resistance to erythromycin and clindamycin, antibiotics frequently given to pregnant women with documented penicillin allergy, has been reported worldwide (6, 24, 158). In support of these findings, the 2002 guidelines issued from the Centres for Disease Control and Prevention (CDC) recommended susceptibility testing of isolates from IAP candidates with penicillin allergy (28). Additionally, increasing resistance rates to erythromycin and clindamycin in both invasive and non-invasive infections among non-pregnant adults have also been described in recent years (9, 24, 83).

Concern has been raised that the use of IAP for prevention of GBS disease could result in increasing incidence of early-onset infections due to microorganisms other than GBS (154), particularly antimicrobial resistant *Escherichia coli* (90). However, a direct assessment of causality between IAP exposure and risk of non-GBS neonatal sepsis has not been established (133), nor has the association between IAP and early-onset sepsis due to ampicillin-resistant *E. coli* (90).

Whereas the improved use of IAP has resulted in a substantial reduction in early-onset GBS disease, it does not prevent late-onset neonatal infections, nor does it address GBS disease in non-pregnant adults (153). New strategies, such as the development of vaccines against GBS, continue to hold the most promise for further prevention of GBS disease. GBS vaccines will be discussed in section 4.

2. Virulence factors

Although GBS usually resides as a commensal microorganism in genital and gastrointestinal tracts, it does have the ability to access several other niches such as the intrauterine compartment and multiple organs. This indicates that GBS has a survival advantage by being efficiently able to adapt to different host environments during the course of infection (146). The development of GBS disease reflects successful bacterial colonization and the capacity to penetrate host physical barriers and requires appropriate expression and regulation of surface-associated and secreted virulence factors that mediate host-cell interactions, including adherence to host epithelial surfaces, invasion across epithelial and endothelial barriers, and interference with innate immune clearance mechanisms (116). Table 1.1 summarizes some key virulence factors of GBS, detailing their proposed pathogenic mechanisms, critical for its ability to cause disease.

This section will address briefly the GBS virulence factors that contribute for the epidemiological characterization of GBS isolates.

TABLE 1.1 Main virulence factors of GBS. Adapted from (44, 108, 137, 146).

Virulence factor	Genes	Molecular or cellular action	Proposed contribution to pathogenesis
Polysaccharide capsule	*cps*A-L *neu*A-D	Impairs complement C3 deposition and activation Masks pro-inflammatory cell wall components Decreases immune recognition, perhaps through molecular mimicry of host sialic acid epitopes	Blocks opsonophagocytic clearance, preventing the recognition of GBS through molecular mimicry of host-cell surface glycoconjugates Delays neutrophil recruitment
β-Haemolysin/cytolysin	*cyl*E	Forms pores in cell membranes Induces inflammatory response and apoptosis Triggers cytokine release	Direct tissue injury Penetration of epithelial barriers Induction of sepsis syndrome Phagocytic resistance Impairs cardiac and liver function
Hyaluronate lyase	*hyl*B	Cleaves hyaluronan	Promotes spread through host tissues during infection Impairment of leukocyte trafficking
C5a peptidase	*scp*B	Cleaves human complement C5a Binds fibronectin	Inhibits PMN[a] recruitment Epithelial adherence and invasion by binding to ECM[b] fibronectin
CAMP factor	*cfb*	Forms pores in host-cell membrane Binds to GPI[c] anchored proteins Binds to IgG, IgM	Direct tissue injury Impairment of antibody function
C protein (α and β components)	*bca* (α) *bac* (β)	Binds epithelial cells Blocks intracellular killing by neutrophils Non-immune binding of IgA	Epithelial cell adherence Epithelial cell invasion Resistance to phagocytic clearance
Alpha-like protein (Alp) family	*bca*, *eps*, *rib*, *alp*2, *alp*3	Binds epithelial cells Suffers antigenic variation as evasion mechanism of antibody detection	Epithelial cell adherence Epithelial cell invasion
Fibrinogen binding proteins A and B	*fbs*A *fbs*B	Binds ECM fibrinogen through repetitive structure motifs	ECM attachment Epithelial adherence Promotes entry of GBS into host cells
Pili	PI-1 PI-2a or PI-2b	Promotes resistance to antimicrobial peptides by an unknown mechanism	Promotes adherence of GBS to host cells

[a] PMN - polymorphonuclear (leukocytes)
[b] ECM - extracellular matrix
[c] GPI - glycosylphosphatidylinositol

2.1 Capsule

The majority of GBS isolates recovered from human infections is encapsulated. The capsule is a major virulence determinant of GBS, being responsible for resistance to opsonophagocitic killing and phagocytosis, as well as for the inhibition of complement system clearance (44).

GBS capsular polysaccharides (CPS) are predominantly composed of repeating units containing four elements: glucose, galactose, N-acetylglucosamine and sialic acid, the terminal sugar on the side chain of all serotypes. Serotypes VI and VIII are an exception to this composition by lacking the N-acetylglucosamine and serotype VIII has an additional ramnose residue (112). The biochemical and immunological properties of the GBS polysaccharide have been extensively studied. In 1987, the role of the GBS capsule in virulence was evaluated in a rat model of neonatal infection, by showing that a non-capsulated mutant of GBS presented significantly reduced virulence as compared to the encapsulated strain (150). The importance of the capsular sialic acid for bacterial evasion of host mechanisms was also demonstrated when an encapsulated strain lost its virulence after removal of the sialic acid in a neonatal rat model of lethal GBS infection (188).

According to the chemical composition, structure, and serological properties, the GBS capsular polysaccharides are classified into nine distinct serotypes: Ia, Ib and II-VIII (112). The polysaccharide capsule is encoded in a gene cluster consisting of a central group of genes that encode serotype-specific glycosyltransferases and polymerases, which are flanked by genes conserved across all serotypes. The genes upstream of the serotype-specific region encode enzymes that synthesize and activate sialic acid, and the genes downstream are hypothesized to function in export of the polysaccharide capsule (32). The capsule gene clusters of all nine GBS serotypes have been described (Figure 1.2) and shown to be both structurally and genetically closely related. Nevertheless, the amino acid sequences of proteins having similar functions in different capsular types were found to exhibit significant heterogeneity (31, 128).

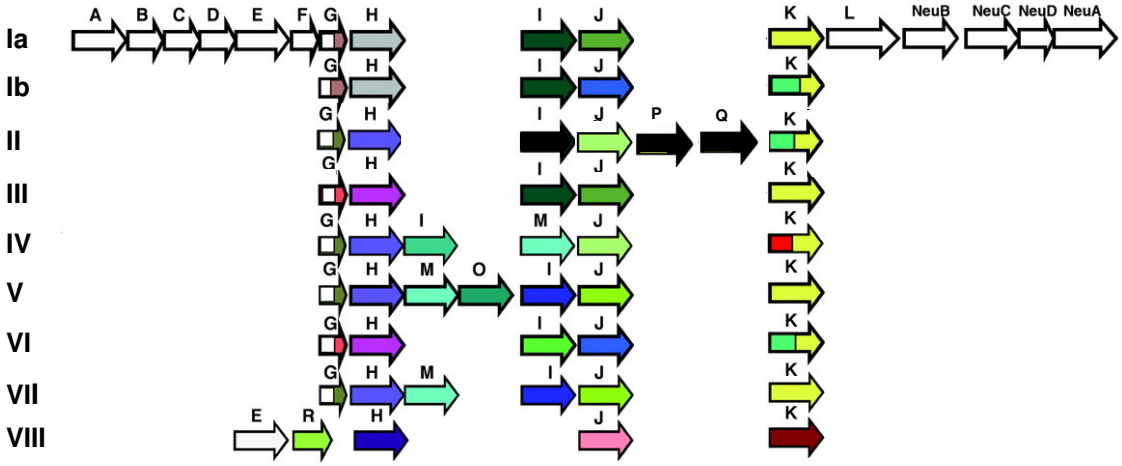

FIGURE 1.2 Capsule gene clusters of the nine GBS serotypes. The colours inside each arrow indicate the degree of similarity of the amino acid sequence to those encoded by other open reading frames (ORFs). Black arrows indicate ORFs with higher BLAST homology to species other than GBS. A gap was introduced between genes *cps*H-*cps*I and *cps*J-*cps*K to permit an alignment of corresponding open reading frames. Adapted from (31, 128).

A detailed examination of the capsule biosynthesis clusters provided evidence for horizontal transfer of capsular genes that may have occurred by intra- and inter-species recombination events, contributing to the genetic diversity of the CPS gene clusters. Furthermore, changes at the capsular locus were proposed to be driven by the equilibrium between the selective pressure imposed by host immunity, that lead to capsular variation, and the conservation of structural elements of a particular capsular polysaccharide that might be required for pathogenicity (18, 31).

2.2 Surface proteins

Surface proteins of GBS are likely to play important roles during different stages of infection, holding promise as vaccine components. Moreover, surface proteins are also interesting for the analysis of some major problems in medical microbiology, such as adherence to epithelial cells, interactions with human extracellular matrix or plasma proteins, and escape from the host immunity response (108).

C protein (α and β components)

Historically, the C protein was the first surface protein antigen identified in GBS. The subsequent characterization of this protein revealed that it was composed of two protein components: the trypsin-resistant α protein and the trypsin-sensitive β protein, both capable of eliciting protective immunity (131). The α-C component mediates invasion of human epithelial cells, by promoting adherence and internalization in epithelial cells (168). The β-C component is a cell-surface receptor which binds the Fc portion of human immunoglobulin A (IgA), interfering with opsonophagocytosis and effective clearance (136). The two components of the C-protein are independently expressed on the bacterial cell surface; a particular strain may express either or both α and β antigens, yet the reason why they are often expressed together remains elusive (108). Furthermore, the phenotypic detection of the α or β protein antigens was considered of limited interest because a significant number of GBS strains does not express either one of them on their surface (84).

Later, sequencing of the α protein gene *bca* revealed the presence of a large region composed of tandem repeating units, identical at the nucleotide level, and defining protective epitopes at the surface of GBS (132). The detection of yet another gene in the same genomic locus of *bca* resulted in the identification of the Rib protein, having an overall similar structure and sequence with the α protein (184), and leading to the recognition of a novel family of bacterial surface proteins, the alpha-like protein (Alp) family.

Alpha-like protein (Alp) family

The Alp family consists of surface associated proteins of similar structure containing repetitive sequences that express protective epitopes. Four members of the Alp family were initially identified: the α, Rib, Alp2, and Alp3 proteins, and two more members, Eps and Alp4 proteins were later included (34). Genetic and immunological evidence indicates that the different Alp proteins are encoded by allelic genes, mutually exclusive, implying that a GBS strain expresses only one member of the family (97).

Partial sequencing of the Alp family members revealed that these proteins have very stable sequences, varying very little in sequence between strains, except for the number of repeats (91). In spite of their stability, these proteins appear to have a chimeric structure, suggesting that the genes can recombine with each other and/or other

genes, and the current proteins known may have been selected for conferring superior fitness (108).

For all members of the Alp protein family, the proteins were shown to vary in size among different clinical isolates, with the size of the protein being proportional to the number of repeats it contains (97). GBS suffers deletions in the repetitive region of the alpha-like proteins by a mechanism of antigenic variation that may provide a means for GBS to evade host immunity. Strains with fewer repetitions are though to have a selective advantage by escaping antibody mediated immunity due to the loss of protective epitopes that result in less antibody binding (67, 111). The average number of tandem repeats of the *rib* gene in invasive GBS recovered from neonates was shown to be smaller than that from adults, implying a distinct contribution of immunity toward this age-related variation (79). On the other hand, naturally occurring variants of Alp containing multiple repeats suggest that longer proteins enhance virulence in humans, probably due to an increased ability to colonize particular niches (68, 70).

Being so, the average number of repeats in an Alp family protein represents the net result of the two conflicting selective pressures, on one hand bacterial virulence that favours proteins with many repeats, and on the other hand, host immunity that promotes selection of variants with fewer repeats. While immune selection results in rapid contraction of the repeat region, release from such selective pressure would allow expansion of the repeat region, which may be required for spread to a new host (108).

2.3 Pili

The presence of pilus-like structures in GBS was first described in 2005 (104). The genes encoding pili in GBS are located within two distinct loci in different regions of the genome, designated pilus-islands 1 and 2 (PI-1 and PI-2), the later presenting two distinct variants, PI-2a and PI-2b (149).

Pili are composed of three subunits: a backbone protein, the *bona fide* pilin, and two ancillary proteins, a pilus associated adhesin and a component that anchors the pili to the cell wall. Both the polymerization and attachment of the pili to the peptidoglycan cell wall occur by sortase-dependent mechanisms (46).

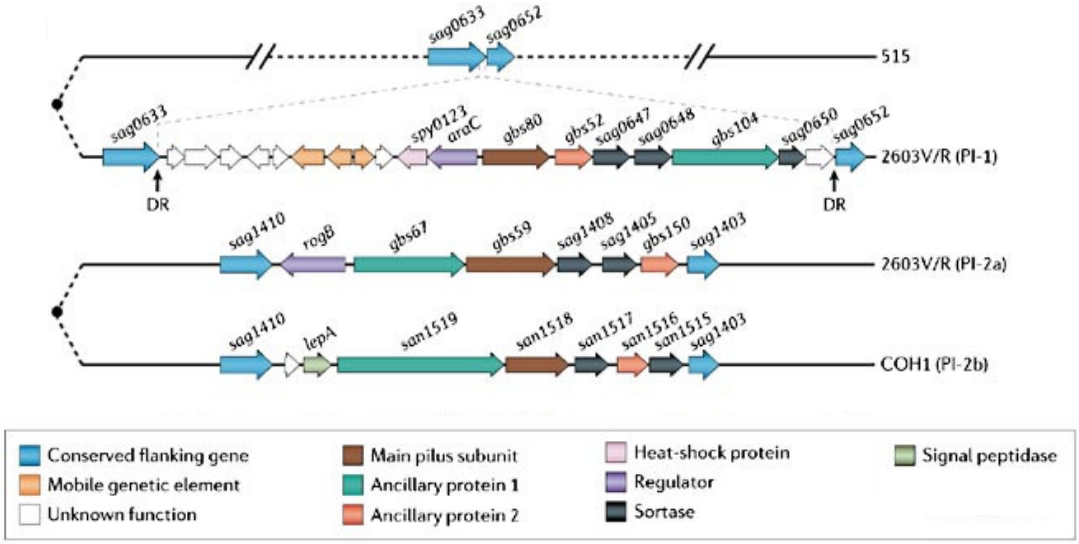

FIGURE 1.3 Schematic representation of PI-1, PI-2a and PI-2b. The upper panel shows PI-1 and the same region in a pilus-negative GBS strain. Adapted from (169).

Even though PI-1 is not present in all GBS strains, PI-2 is ubiquitously expressed. The sequences of the all three pilus-islands appear to be remarkably well conserved, with PI-2a being the only island to show some extent of variability (126). Nevertheless, and considering the variability observed in other pathogenic bacteria, it has been suggested that the expression of pili may be regulated in order to have a transient expression at the bacterial surface, thus avoiding the selective pressure of the host immune system. Another possible explanation is that the main environmental niches that GBS occupies in humans (lower gastrointestinal tract and vaginal mucosa) are relatively inert from an immunological standpoint, not providing a sufficient immune pressure to drive significant variability (126).

While a specific function for pili has not been identified, these structures have been recognized to play a role in adherence, invasion and translocation of epithelial cells (94, 117, 143), and also as important for biofilm formation, all functions possibly conditioning tissue-tropism (119, 148).

3 Antimicrobial resistance in GBS

3.1 β-lactams

Penicillin and other β-lactam antibiotics covalently bind to and inactivate the bacterial proteins (penicillin-binding proteins, PBPs) involved in the formation of peptidoglycan by crosslinking its subunits in the bacterial cell wall. While in *Streptococcus pneumoniae* penicillin resistance involves the stepwise accumulation of mutations in the PBPs or, more frequently, the acquisition of foreign DNA that alters the PBP genes; in GBS, both invasive and colonizing isolates are considered universally susceptible to penicillin and ampicillin, and these are the first choice antibiotics for intrapartum prophylaxis as well as for treatment of GBS infections in all age groups (36). However, two recent studies have described reduced penicillin susceptibility in isolates recovered from both non-invasive and invasive infections. These isolates had mutations in a penicillin-binding protein (PBP-2x) resulting in increased MICs to penicillin (36, 89). While these findings raise concerns as to the possibility of emergence of penicillin resistance in GBS, their clinical impact is yet to be defined and, since these isolates have got to be found in significant numbers, they remain anecdotal cases.

3.2 Macrolides

Macrolides inhibit bacterial protein synthesis by binding to a specific location of the 50S subunit of the bacterial ribosome, thereby preventing its association to tRNA. Resistance to macrolides and related antibiotics in GBS presents as resistance to erythromycin alone or to both erythromycin and clindamycin, and occurs mainly by two mechanisms. The first involves target-site modification by a methylase encoded by an *erm* (erythromycin ribosome methylase) gene. The methylation of 23S rRNA at the erythromycin-binding nucleotide target alters the binding site for antibiotics on the ribosomes, thereby conferring broad-spectrum resistance to macrolides, lincosamides and streptogramin B (MLS_B phenotype). Expression of MLS_B resistance is either constitutive ($cMLS_B$) or inducible ($iMLS_B$). Inducible resistance occurs when the presence of an inducer antibiotic is necessary to allow the proper translation of the mRNA encoding the methylase. By opposition, in constitutive expression, the

methylase is produced even in the absence of an inducer antibiotic (105). In the second mechanism a membrane-bound pump, encoded by a *mef* (macrolide efflux) gene, is responsible for the active efflux of the antibiotic, causing resistance to 14- and 15-membered ring macrolides while retaining susceptibility to lincosamides and streptogramin B (M phenotype) (105).

In GBS the spread of genes belonging to the *erm*(B) class (23, 24, 58, 60, 64, 83, 158) and, less frequently, to the *erm*(TR) subset of the *erm*(A) class (38) account for the vast majority of macrolide resistance. Moreover, most of these studies report an association between the *erm*(B) gene and $cMLS_B$ phenotype, while others describe also the association of $iMLS_B$ and the *erm*(TR) gene (24, 151). An additional *erm* gene, the *erm*(T), which had only been described in *Streptococcus bovis*, was recently found among $iMLS_B$ GBS strains (41). Among the isolates presenting the M phenotype, the *mef*(A) and *mef*(E) genes are responsible for macrolide resistance (24, 127). Despite the fact that antimicrobial resistance varies according to the geographical location, there has long been a wide consensus as to the association of serotype V and erythromycin resistance (24, 182).

In Canada, a GBS isolate that was susceptible to erythromycin but resistant to clindamycin was shown to carry the *lin*(B) (or *lnu*B) gene, responsible for lincosamide nucleotidylation and previously described in *Enterococcus faecium* to confer resistance to lincosamides only (38). This resistance determinant was later found in Korea and suggested to be co-localized in the same mobile genetic element as the *erm*(B) gene (158). In contrast, GBS strains from New Zealand were found to be simultaneously resistant to lincosamide and streptogramin A, giving rise to the proposal of a new LS_A phenotype (118). The presence of the LS_A phenotype in different GBS clones suggested a horizontal source of the resistance determinants, although a conjugative transfer of resistance traits could not be detected. Additionally, no known genes conferring resistance to lincosamides or streptogramin A antibiotics were found (118). This phenotype has also been detected in Taiwan (183) and Korea (177).

3.3 Tetracycline

Tetracycline inhibits bacterial protein synthesis by preventing the association of tRNA with the bacterial ribosome (30). In GBS, resistance to this antibiotic is nearly

ubiquitous and is most frequently due to a protein encoded by *tet*(M) or *tet*(O), which protects the ribosome from the action the tetracycline. Less common *tet*(K) or *tet*(L) genes encode a tetracycline efflux pump (30).

Whereas *tet*(M) is the most frequent tetracycline resistance determinant among GBS isolates recovered from human infections, *tet*(O) is common among those bacteria isolated from bovines (42, 47). Other unusual ribosomal protection genes *tet*(T) and *tet*(W) have also been found among GBS isolates from human infections (151).

Tetracycline is not usually used for the treatment of streptococcal infections. However, surveillance of tetracycline resistance is important because the genetic elements carrying *tet* genes are often the same that carry genes encoding resistance to macrolides, lincosamides and chloramphenicol (30). An association between the macrolide resistance gene *erm*(B) and either the presence of *tet*(M) in human or *tet*(O) in bovine isolates was found, supporting the importance of horizontal gene transfer for antimicrobial resistance evolution in GBS (47). Even though many studies have found an association between *tet* and *erm* genes pointing to their carriage by the same transposons and also suggesting the epidemic spread of resistant clones (64, 145, 151), in others tetracycline resistance was not linked to erythromycin resistance, indicating an independent dissemination of resistance (35).

3.4 Quinolones

The quinolones target DNA gyrase and topoisomerase IV, which are involved in the supercoiling of bacterial DNA that is essential to cellular processes such as DNA replication and transcription. Resistance to fluoroquinolones arises mainly due to target gene modification by mutation, yet the acquisition of a single mutation does not usually confer significant fluoroquinolone resistance. Rather, resistance is a cumulative process, with increasing numbers of mutations generally correlating with higher MICs. Thus mutations that confer fluoroquinolone resistance occur in internal regions called the quinolone resistance-determining region (QRDR) of the genes *gyr*A and *gyr*B (encoding the gyrase subunits) and *par*C and *par*E (encoding the topoisomerase IV subunits). While *gyr*A and *par*C mutations are frequent, changes in *gyr*B and *par*E also occur but are less common (189).

GBS were uniformly susceptible to quinolones until 2003, when three isolates highly resistant to multiple quinolones were described in Japan, due to double-point mutations in the QRDR of *gyr*A and *par*C (88). Also in a report from Japan, high resistant rates to quinolones were later observed, particularly among invasive disease in non-pregnant adults (31.4%) and significantly associated to serotype Ib (134). Another survey from the United States identified 5% of the isolates as being resistant to levofloxacin, and suggested that healthcare-associated spread had occurred, as an association with prior quinolone therapy was observed (185).

3.5 Chloramphenicol

Chloramphenicol inhibits protein synthesis by specifically blocking the 50S subunit of the bacterial ribosomes. Resistance to this antibiotic is generally due to the synthesis of the enzyme chloramphenicol acetyltransferase, encoded by the gene *cat*, which inactivates the antibiotic by chemical conversion, losing the ability to bind to the ribosomes. In GBS, both chromosomally integrated through transposons and plasmid carried *cat* genes have been detected (173).

4. Vaccines

Development of a vaccine as a prophylactic measure is the most promising approach to the prevention of GBS infections, given the potential adverse effects of IAP discussed previously, as well as the need for effective prevention of LOD and invasive disease in non-pregnant adults (48, 187).

The rationale for the development of a GBS vaccine was experimental and clinical evidence that specific antibodies directed against surface structures of the bacteria are protective (187). However, it is important to note that most pregnant women (85-90%) lack protective antibodies at the time of delivery (90). A maternal GBS vaccine given before or during pregnancy could confer immunity to peripartum maternal infection as well as passive immunity to the newborn due to efficient transport of protective IgG antibodies through the placenta (156, 187). Additionally, it is thought that immunization of pregnant women and their infants can have a herd effect, providing additional health benefits among non-vaccinated members of a population, particularly the adults at high risk for GBS disease (159).

The investigation regarding immunization against GBS goes back to the 1960s, when Rebecca Lancefield showed that capsular polysaccharide (CPS) specific antibodies have a protective role for GBS infection in mice (102). Further studies revealed that immunization with purified CPS alone was safe and well tolerated, however insufficiently immunogenic, suggesting that conjugation with a carrier protein might elicit better immune responses to the capsule polysaccharides (5).

The first GBS polysaccharide-protein conjugate vaccine tested in humans coupled a type III polysaccharide with tetanus toxoid (TT), and proved to be safe and immunogenic in pregnant women (87). However, the lack of cross reactivity between different serotypes revealed that individual serotypes only offered protection to homologous capsular types, driving the design of a multivalent conjugated vaccine that would provide a broader coverage (187). A vaccine formulation based on independent conjugation of GBS capsular polysaccharides Ia, Ib, II, III and V to TT, plus a bivalent (II and III) preparation, undergone phase I and II clinical trials. All the formulations revealed safety and high immunogenicity in healthy adults, and the co-administration of two conjugates did not suffer from immune interference (141). Later, immunization of healthy elderly persons with a GBS serotype V-TT conjugate vaccine elicited protective

type-specific antibodies, suggesting the potential for vaccination to prevent the increasing number of invasive GBS infections observed in this age group (140).

A major problem associated to a CPS-based vaccine is the choice of serotypes to include in such a vaccine. In the United States, a vaccine against serotypes Ia, Ib, II, III, and V is thought to offer protection to 80-95% of the population including neonates, pregnant women and non-pregnant adults (75, 95, 160, 176). Although this vaccine would likely provide coverage against pathogenic serotypes that are currently causing disease in the United States and most European countries, it does not offer protection against serotypes that are more prevalent in other parts of the world. Serotypes VI and VIII are predominant among Japanese women (98), serotype IV is the most frequent in the United Arab Emirates (3) and also in some European countries a significant prevalence of other serotypes has been reported, such as Ib among invasive isolates in Ireland (45) and colonizing isolates in Germany (15). Whereas the prevalence of particular serotypes has been shown to vary geographically, there are also significant discrepancies over time in the same region. In the United States, serotype V was not recognized as an important serotype causing GBS invasive disease until the late 1990s (14), and there is recent work reporting the emergence of serotype IV (40). Furthermore, non-typeable (NT) isolates can account for up to 29% of colonizing (81) and 15% of invasive isolates (176). Whether these isolates express an unknown capsule or no capsule at all, they will not be covered by the current formulations of polysaccharide-based vaccines.

Other concerns regarding polysaccharide-based vaccines are serotype replacement and capsular switching. Serotype replacement due to vaccines has been observed in recent studies of *S. pneumoniae*, with the expansion of particular serotypes that were not included in the pneumococcal vaccine following a decrease in the infections caused by vaccine serotypes (1). Additionally, capsular switching was thought from the beginning to be a mechanism that could erode the benefits of pneumococcal vaccination programs since vaccination could provide the selective pressure for virulent genotypes to switch capsules and escape vaccine induced immunity (22). It is possible that similar responses will be seen with a future introduction of GBS vaccination (159). Changes in the prominence of various GBS serotypes over time have provided an additional challenge to vaccine development and may dictate reformulation of a multivalent vaccine. The need to overcome serotype-dependence drove the search

for other surface antigens, and some surface proteins revealed potential to elicit protective immunity, namely the α- and β-antigens of protein C (69, 113), C5a-peptidase (29), as well as the Rib (103) and Sip (20) proteins. However, due to the fact that most of these proteins are not conserved at the gene level in all GBS isolates, additional efforts have been directed towards identifying bacterial surface proteins that, combined in a single vaccine, could provide serotype-independent protection against GBS (115).

The identification of pilus-like structures on the GBS surface (104), their potential importance in GBS pathogenesis and the fact that these are ubiquitously expressed rendered these structures important candidates for the development of a universal GBS vaccine that could be potentially capable of preventing GBS disease in all age groups. In a recent study, the offspring of mice immunized with a combination of components of the three known pilus-islands was protected against challenge by GBS strains of all serotypes. Furthermore, the role of pili in promoting bacterial adherence to host tissue suggests that pilus-based vaccines might also elicit antibodies capable of preventing colonization (126).

5. Characterization of GBS isolates

Bacterial epidemiologists use typing methods to study the dissemination and population dynamics of human bacterial pathogens in clinical and environmental settings, including their transmission patterns and the identification of risk-factors for the control of infectious diseases in human populations (179).

5.1 Phenotyping methods

Bacteriologists have long used phenotypic typing methods to group microorganisms according to their similarity in observable traits (phenotypes), which in turn result from the expression of their genotypes (179). Conventional phenotypic methods include serotyping (based on differences in surface epitopes), phage-typing (based on resistance to infection by a standard set of bacteriophages), biotyping (according to the different metabolic capabilities of the cell), bacteriocin typing (the presence or susceptibility to a specific group of bacteriocins), and antibiotic resistance typing (susceptibility to a panel of antimicrobials). Although providing convenient means to identify outbreak isolates in the short term, such methods are in general inadequate for evolutionary studies and increasingly recognized not to afford sufficient resolution (56).

Serotyping

For GBS, serotyping is the most commonly used method of phenotypic assessment. It is based on the expression of distinct polysaccharide capsules at the bacterial surface that react with serotype-specific antibodies. There are nine different serotypes known: Ia, Ib and II-VIII (156). A new provisional serotype IX was proposed in 2007 (161); however, the inexistence of a commercially available sera renders this serotype unaccountable in the phenotypic characterization of GBS. Moreover, a significant number of strains lack detectable capsule polysaccharide, being considered non-typeable (NT). Whether these strains do not express a capsular polysaccharide (non-encapsulated) or express polysaccharide variants that fail to react with available sera, they are not discriminated by the methodologies used (7).

Antimicrobial susceptibility testing

The discrimination of strains based on antimicrobial susceptibility testing is dependent on the diversity, stability and relative prevalence of the detectable resistance in study isolates (179). In clinical practice, most microbiology laboratories perform antimicrobial susceptibility tests in automated systems, since its results are useful in guiding clinicians in selecting appropriate therapy.

For research purposes, susceptibility testing of selected antibiotics is usually performed by the Kirby-Bauer disk diffusion method. Small disks pre-impregnated with a standard concentration of antibiotic are placed onto a plate upon which bacteria are growing. After incubation, the diameter of inhibition around the disk can be compared to reference tables to determine whether the bacterial isolate is susceptible, intermediately susceptible, or resistant to the antibiotic. A variation on this approach is to use a strip impregnated along its length with gradual concentrations of antibiotic (E-test), which after incubation creates an ellipse shaped zone of no growth. The minimum inhibitory concentration (MIC) can be read from the concentration marking where the ellipse meets the strip (179). Additionally, detection of resistance phenotypes by a disk approximation method (D-test) can be performed. Methods and interpretative criteria follow recommendations from international and independent organizations, such as the Clinical and Laboratory Standards Institute (CLSI) (33).

5.2 Genotyping methods

Genotypic typing methods assess variation in the genomes of bacterial isolates with respect to composition (presence or absence of plasmids or mobile genetic elements), overall structure (restriction endonuclease profiles, number and position of repetitive elements), or precise nucleotide sequence (of genes or intergenic regions) (178).

Restriction Fragment Length Polymorphism (RFLP)

This method is based on DNA digestion with one or more endonucleases. The resulting restriction pattern of variable length fragments is obtained by separation in conventional electrophoresis and reflects the frequency and distribution of endonuclease recognition sites (129). The DNA fragments can be further transferred to a membrane

and hybridized to a probe, allowing additional subtyping of the isolates (Southern blot). The fingerprint obtained is dependent on the recognition of fragments carrying DNA sequences identical to the probe. The choice of the probe is critical for the discriminatory power and typeability of the method (129).

In GBS, RFLP was used to study the population structure of serotype III isolates, discriminating three distinct phylogenetic lineages, and further identified a particular one associated with invasive disease in neonates, suggesting that this lineage could have increased virulence (166). RFLP and Southern blot have also proved helpful in classifying non-typeable isolates and effective as a tool for subtyping GBS based on insertion sequences (167).

Pulsed-field gel electrophoresis (PFGE)

PFGE involves the exposure of chromosomal DNA to endonucleases that recognize only a few sites in the bacterial genome, generating macrorestriction fragments. Agarose plugs containing the digested chromosomal DNA are inserted into the wells of an agarose gel, and the restriction fragments are resolved into a pattern of discrete bands by an apparatus that periodically switches the orientation of the electric field across the gel. The DNA restriction patterns of the isolates are then compared with one another to determine their relatedness (129, 170).

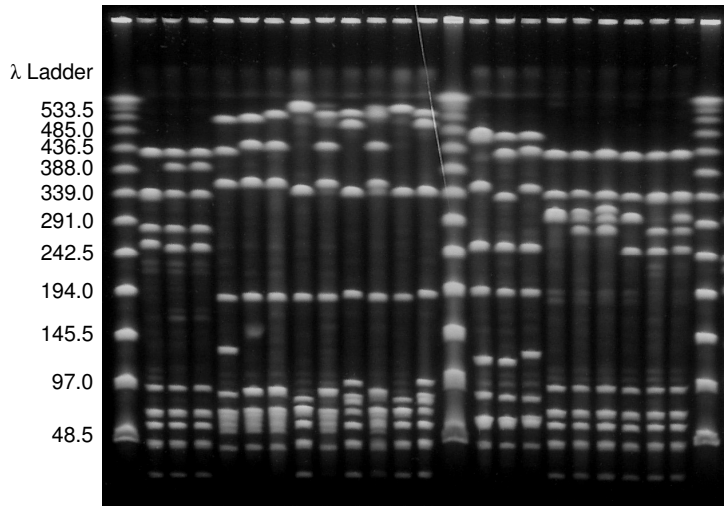

FIGURE 1.4 PFGE profiles generated following SmaI digestion of GBS chromosomal DNA. Fragment sizes (Kb) of the Lambda (λ) PFGE marker (New England Biolabs, Beverly, MA) are presented.

PFGE's potential value in molecular epidemiologic studies of GBS infections was first described when compared to conventional electrophoresis. PFGE was shown to yield reproducible and easily readable profiles, and had enough sensitivity and discriminatory power for appropriate evaluation of the genetic relatedness of strains within a serotype and from different serotypes (55, 66).

However, manual analysis and comparison of large numbers of PFGE profiles was difficult. The need to translate these data into epidemiologically useful information led to the proposal of interpretative criteria, as follows: isolates are categorized as closely related if one genetic event (usually a point mutation or an insertion or deletion of DNA) generates two to three band differences in their PFGE profiles; possibly related if two independent genetic events generate four to six band differences; or unrelated if the PFGE patterns differ by changes consistent with three or more independent genetic events (generally seven or more band differences) (170). Development of sophisticated software programs such as Bionumerics (Applied Maths, Sint-Martens-Latem, Belgium) has allowed the comparison of numerous collections of isolates by normalization of the band patterns over multiple gels and consequent construction of dendrograms from which phylogenetic relationships can be assessed.

PFGE has been extensively used in epidemiological studies of large populations of GBS isolates, and on evaluation of population dynamics of GBS within restricted cohorts. It has been particularly relevant in addressing questions of transmission and acquisition of GBS, by demonstrating that newborns and young infants usually carry the same GBS clone as their mothers (72); that recurrence of GBS disease in infants can be associated with either the original infecting strain or a second acquired strain (71); and that sex couples frequently share the same strain, which is likely to be transmitted by sexual contact (120).

Multilocus sequence typing (MLST)

MLST is a sequence-based typing method that involves sequencing of internal fragments of seven housekeeping genes. The sequences are then compared with known alleles deposited at the MLST database (http://pubmlst.org/sagalactiae), and an allele number is assigned to each sequence, generating an allelic profile. Each isolate is therefore characterized by an allelic profile, a seven-integer number that can also be designated by a sequence type (ST) (86). MLST offers a valuable tool for the

characterization of bacterial strains. The major advantage of MLST over PFGE is the precise, unambiguous and portable nature of the data obtained, so that the isolates typed in one laboratory can be rapidly compared with all previously typed strains (56).

In recent years, MLST became increasingly used for the characterization of bacterial populations because of its ability to infer levels of relatedness between strains and the reconstruction of evolutionary events (57). These questions have been addressed based on an algorithm, eBURST, that divides an MLST data set into groups of related isolates by implementing a simple model of clonal expansion and diversification (57).

This model predicts that the emergence of clonal complexes (CCs) is due to an increase in the population of the frequency of the founding genotype, as a consequence of either a fitness advantage or of random genetic drift. This genotype increases in numbers and by gradual diversification (point mutation or recombination) gives rise to a clonal complex. In terms of MLST, the descendants of the founder allelic profile will initially remain unchanged, but over time variants in one of the seven alleles will arise. These genotypes, which have allelic profiles that differ from that of the founder at only one of the seven MLST loci, are called single-locus variants (SLVs). Eventually, SLVs will diversify further, to produce variants that differ at two of the seven loci, called double-locus variants (DLVs), and so on. Within each clonal complex, eBURST displays links between STs that correspond to an hypothetical pattern of descent (57).

However, due to the fact that the eBURST algorithm is locally optimized, which can result in links within the clonal complexes that violate the rules proposed, a globally optimized implementation of the algorithm was proposed, goeBURST (62). This solution provides the representation of the level of tiebreak rule reached before deciding if two STs should be linked, which can be used to visually evaluate the reliability of the represented hypothetical pattern of descent. A software implementation of the goeBURST algorithm is available at http://goeBURST.phyloviz.net (62).

PCR-based gene profiling

Polymerase chain reaction (PCR) is a nearly universal typing method, with several applications in bacterial typing systems, and exhibits an easily adjustable level of discrimination. Its major advantages include high reproducibility, technical simplicity, wide availability of equipment and reagents, and rapid turnover time (179).

Several PCR-based typing systems have been used to genotype GBS isolates and include, among other, molecular serotyping (92, 128), sub-typing within particular serotypes (121), surface protein gene profiling (34), detection of mobile genetic elements (93), and of antimicrobial resistance genes (165).

Whole-genome sequences comparisons

The development of efficient and less expensive sequencing methods has produced a significant number of complete genome sequences of pathogenic microorganisms in recent years. The comparison of whole-genome sequences offers the possibility to assess genetic differences within a bacterial species, providing insights on how genetic variability drives the evolution of virulence mechanisms.

The first complete GBS genome sequences were made available in 2002 (65, 172). The GBS genome is nearly 2.2 Mbp long and has over 2100 predicted coding regions. Both studies revealed substantial similarity with the genomes of the related human pathogens *Streptococcus pyogenes* and *S. pneumoniae*, representing a conserved backbone between streptococcal species. On the other hand, GBS differed from other streptococci in genome regions containing known and putative virulence genes, mostly encoding surface proteins and genes related to mobile elements, suggesting that these regions could be considered as pathogenicity islands (65). These genes were probably related to adaptation of GBS to distinct niches in its human and animal hosts and were expected to play a role in colonization or disease. Accordingly, the high number of genes associated with mobile genetic elements, including bacteriophages, transposons and insertion sequences supported the acquisition of virulence traits from other species (172).

Comparative analysis of multiple genomes introduced the concept of a “pan-genome”, consisting of a core genome shared by all isolates, accounting for approximately 80% of any single genome, and involved in housekeeping and regulatory functions, plus a dispensable genome consisting mostly of strain-specific genes. Again, the abundance of genes associated with mobile and extrachromosomal elements found in the variable portion of the genome, supported the hypothesis that the acquisition of the majority of strain specific traits depends on lateral gene transfer (171).

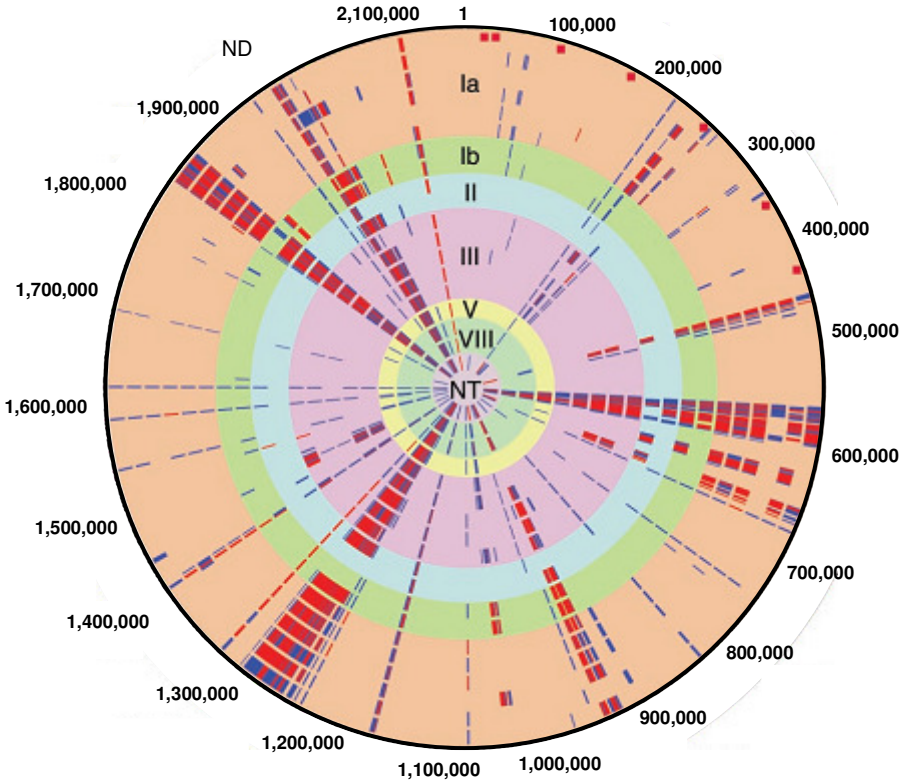

FIGURE 1.5 Circular representation of GBS genomes by comparative genomic hybridization. GBS strains of serotypes Ia, Ib, II-V, VIII and NT are represented. Red bars inside the circle represent genes absent from the strain; blue bars represent genes with ambiguous hybridization result. Numbers outside the circle represent the scale in bp. Adapted from (172).

Furthermore, genetic heterogeneity among GBS strains, even of the same serotype, revealed that evolution within genes encoding surface and secreted proteins and those involved in the biosynthesis of the capsule is mainly due to recombination events leading to gene acquisition, duplication, and reassortment with the consequent replacement of several genes or to the allelic exchange within a particular gene. These processes allow GBS to express various combinations of virulence factors, which are likely to serve as means of adapting to host immunity (18, 172). Integrative conjugative elements are also thought to play a role in the evolution of the GBS genome through their plasticity and potential for gene acquisition and mobilization, as well as exchange with other species (17).

Finally, it was demonstrated that GBS strains can exchange DNA segments of up to 334 Kb of the chromosome, probably through mobilization by conjugative

elements. The analysis of the contribution of such recombinational exchanges to the dynamics of the GBS chromosome, has led to the proposal of an evolutionary model in which clonal complexes of clinical importance are derived from a single clone and evolved through the exchange of large chromosomal regions with more distantly related strains (19). This study, in agreement with previous observations that the GBS chromosome is a mosaic of large chromosomal fragments from different ancestors (171), suggests that particular combinations of genes could be horizontally transferred together. If advantageous fitness is achieved by specific combinations of genes that increase the ability to cause disease or persist through asymptomatic colonization, these beneficial alleles would not be randomly distributed among population members, but found more often than expected (i.e., at linkage disequilibrium). If this is the case, associations of specific alleles within the GBS lineages, including housekeeping (MLST) and virulence genes, as well as a new distribution of endonuclease recognition sites generating PFGE patterns, can be investigated within GBS populations.

5.3 Molecular epidemiology of GBS

Molecular epidemiology has been used to discriminate genetic lineages in order to probe for associations between specific GBS genotypes and disease. DNA-based typing methods such as PFGE and MLST are now extensively used in molecular typing of GBS isolates, alongside with classical serotyping and antimicrobial susceptibility testing.

Serotype distribution

Serotyping remains an important methodology in epidemiological studies evaluating fluctuations of different GBS clones in the human population (14, 78, 98). Serotypes have been shown to vary geographically and over time, which may have a serious impact on a future vaccine formulation. Additionally, the prevalence of the GBS serotypes is age-dependent and different among colonizing and invasive isolates.

A recent review of the GBS seroprevalence in pregnant women in the United States as compared to other geographic locations revealed substantial differences (81). While back in the 1990s most studies from the United States described serotype V as the most frequent among colonizing isolates, serotypes Ia and III have become more

prevalent in recent years. In Canada all these three serotypes were similarly prevalent and in Europe serotype III is dominant in most countries (81), excluding Greece (175), and Germany where serotype Ib is emerging (15). In a recent review, three African countries reported serotypes III and V as being equally relevant (81). In Asian countries, after serotype III, serotype Ib and serotype V are equally frequent (81), but in the particular case of Japan serotypes VI and VIII are predominant among pregnant women (98), and these are serotypes not frequently detected elsewhere. Also in the Middle-east serotypes Ia, II, III and V have similar prevalence (81), but in the United Arab Emirates, serotype IV is the most frequent, again an infrequent serotype (3).

An obvious assumption is that major differences in the serotype prevalence of GBS isolates colonizing pregnant women will be reflected on vertical transmission, therefore on the isolates that cause invasive disease in their newborns. Yet this is not the case, since serotype III is by far the most frequent serotype among neonatal invasive isolates in all continents (8, 114, 134, 144, 190). These observations led to the suggestion of an enhanced invasive potential of this serotype, resulting in its higher representation in neonatal invasive disease that has been shown to be mainly attributable to a particular genetic lineage of serotype III, which will be discussed later in this section. Furthermore, considering the significant decrease observed in EOD cases upon wide application of antimicrobial prophylaxis, as opposed to no remarkable changes in LOD cases, it is possible that different serotypes from sources other than maternal colonization will emerge in coming years.

In the past two decades GBS has been increasingly associated with invasive disease in non-pregnant adults, particularly the elderly (9, 130, 144, 160). However, the epidemiology of GBS in infections among adults is less well known than that of neonates and pregnant women, and information on colonizing isolates is nearly residual in these populations, probably reflecting the recent emergence of GBS as an important pathogen in adults.

Among the isolates causing invasive infections in non-pregnant adults, serotype V is the most frequent in North America (144, 160, 176) and Europe (99), even though in Norway a recent report describes both serotypes V and III as equally frequent (8), which was also recently observed in Korea (158). A main exception is again Japan, where serotype Ib is the most prevalent among invasive disease in non-pregnant adults (134). Serotypes VI and VIII are less commonly found as causes of invasive disease

than of maternal colonization in Japan (134) but have been detected elsewhere, together with serotype VII (158, 160, 190).

PFGE and MLST-based genetic lineages

Molecular methods have provided new clues about the population structure of GBS, particularly the recognition of genetic lineages within serotypes that were shown to differ in virulence potential and tropism. Among serotype III isolates, two main genetic lineages have been identified based on MLST. While ST19 is more frequently found among colonizing isolates (86, 151), ST17 has been mostly recognized as a hypervirulent clone, and strongly associated with neonatal invasive infections (12, 86, 107, 109, 124), even though not all studies are in agreement with the correlation between this ST and increased pathogenic potential (37). The potential application of MLST to infer the phylogenetic evolution of GBS has led to the proposal of a bovine origin for ST17 (11, 77), yet this issue remains controversial (18, 162). In Hong Kong another serotype III genetic lineage (ST283) prevails among isolates recovered from invasive disease in non-pregnant adults (80). Considering that ST17 and ST283 do not have in common any of the seven MLST alleles, these lineages are likely unrelated from a recent evolutionary standpoint. ST17 and ST283 are causing infections almost exclusively on neonates and adults, respectively, and ST17 is worldwide spread while ST283 is apparently confined to a geographic location. These observations support for a distinct evolution of these lineages while sharing the same capsular type III, with ST283 likely being more recent, and probably account for their particular success and tropism.

Other MLST-based genetic lineages have been described as dominant among GBS isolates causing invasive disease, particularly serotype Ia/ST23 and serotype V/ST1 (8, 109, 124, 164). Despite the fact that ST1 is the dominant genetic lineage among serotype V GBS isolates in Europe and the United States, other countries have reported a major prevalence of other lineages. ST26 is more frequent in Senegal and Central African Republic (16) and has also been found in Brazil (139). Again, the fact that ST1 and ST26 share only three out of the seven MLST alleles suggests a separate evolution of these lineages within serotype V, to which local factors can also contribute, determining emergence of regional successful lineages.

Although the distinction of lineages within a particular serotype has proved useful, a complete correlation between capsular type and the genetic lineages as defined

by PFGE and MLST was never found (18, 109, 122). The serotype-independent clustering of strains implies that the diversification of the GBS populations is ongoing. These observations support the hypothesis that closely and divergently related clones share the genes coding for a particular capsular type, suggesting that capsular switching probably occurs in GBS (37, 86, 109, 122).

PFGE is a whole-genome based technique, yet of low resolution, and MLST relies on housekeeping genes, not taking into account the variable genome. Consequently, methodologies based on identification of putative virulence genes from the variable portion of the genome, such as those encoding the alpha-like surface proteins and pili, could be of further assistance for differentiation of the GBS populations. The presence of particular surface protein genes has been often correlated with the capsular type (Table 1.2) and it has been proposed that such associations may represent evolutionary lineages, considering that immune selection has probably favoured certain combinations of surface structures because of their greater fitness (108).

TABLE 1.2 Association between Alp surface protein genes and serotypes.

Serotype	Alp surface protein gene	References
Ia	*bca*, *eps*	(108, 112, 142, 164)
Ib	*bca*	(108, 112, 142, 164)
II	*bca*, *rib*	(108, 112, 142)
III	*rib*	(108, 112, 142, 164)
IV	*bca*	(40)
V	*alp*3	(108, 112, 142, 164)
VII	*alp*3	(164)
VIII	*alp*2, *alp*3	(108, 112, 164)

The complement of surface protein gene profiling to serotyping and MLST resulted in an overall good correlation of typing methods, which in combination allow the discrimination of otherwise undistinguishable clones. Up to now, the genetic lineages more frequently found among GBS isolates are III/ST19/*rib*, III/ST17/*rib*, Ia/ST23/*eps*, Ib/ST9/*bca*, II/ST28/*rib* and V/ST1/*alp*3 (64, 151, 164), this last one significantly associated to erythromycin resistance (64, 151).

The epidemiological characterization of GBS populations has revealed a large number of genetically distinct lineages, most capable of both asymptomatic colonization and of causing invasive disease in different age groups. The stability and dominance of

a few worldwide disseminated lineages that are responsible for the majority of infections regardless of continuous antibiotic and immune selective pressures, suggest that they are extremely well adapted to their particular niche.

Nevertheless, the prevalence of serotypes and genotypes among different human populations is not the same, suggesting that particular lineages may be better adapted to particular host characteristics, including age. Furthermore, the occurrence of infrequent clones as causes of invasive infections in specific regions raises the possibility that lineages with enhanced invasiveness may be emerging and expanding at a regional level. Continuous surveillance is crucial for a better understanding of the dynamic nature of GBS populations.

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CHAPTER 2

Analysis of group B streptococcal isolates from infants and pregnant women in Portugal reveals two lineages with enhanced invasiveness

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Summary

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 only associated 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.

Introduction

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, were 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 of 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 overrepresentation of serotype III, ST17, among neonatal invasive isolates was confirmed, and this lineage was responsible for almost half of the cases of LOD. In contrast, a lineage expressing serotype Ia and presenting ST23 and ST24 was also found to have enhanced virulence but was mainly associated with EOD.

Materials and Methods

Bacterial strains

GBS carriage isolates (n=269) were recovered from vaginorectal swabs of healthy asymptomatic women in their last trimester of pregnancy, using the recommended procedures for enhanced recovery of GBS (9). The bacteria were isolated during the normal antenatal follow-up of women in the Lisbon area from 2002 to 2004. Invasive GBS isolates (n=64) were recovered from blood or cerebrospinal fluid (CSF) of infants up to 3 months of age. From 2000 to 2002, only two laboratories from tertiary hospitals in the Lisbon area participated in the survey, and in 2003 and 2004, an additional nine laboratories geographically scattered in Portugal joined the survey. Accordingly, the majority of the isolates were recovered in 2003 and 2004 (n=45/64). The results of the preliminary characterization of 17 invasive isolates recovered from 2000 to 2002 in one of the hospitals were reported previously (16). The laboratories were asked to submit all nonduplicate GBS isolated from normally sterile sites. Whenever isolates were available from blood and CSF of the same patient, only the CSF isolate was included in the study. Isolates were identified to the species level by Gram stain, colony morphology, catalase test, and a commercial latex-agglutination technique (Slidex Strepto B, bioMérieux).

Serotyping, Pulse-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST)

Capsular serotyping was done by slide agglutination using sera for types Ia, Ib, and II to VIII (Hemolytic Streptococcus 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 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 (UPGMA) dendrograms. The Dice similarity coefficient was used with optimization and position tolerance settings of 1.0 and 1.5, respectively. Clones were defined as groups of isolates ($n \geq 3$) presenting profiles ≥80% related on the dendrogram, as previously described for *Streptococcus pneumoniae* (33). The choice of this cut-off value for the definition of clones is

supported by prior work in 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 (35), as well as with those defined using other typing methods (6, 7).

The characterization by MLST of selected isolates was performed as described previously (22). At least one isolate of each PFGE clone was characterized by MLST. In larger PFGE clones, isolates representing both colonization and infection and also the different serotypes grouped in the same PFGE cluster, were characterized by MLST. Whenever an invasive isolate of a particular serotype was not grouped into a PFGE clone, the isolate was characterized by MLST. eBURST software (14) and the entire GBS MLST database (http://pubmlst.org/sagalactiae/) were used to define relationships between STs. A recently described framework was used to analyze the relationships between the results of the typing methods (7). Fisher's exact test (two-tailed) was used to test associations and a *P* value of <0.050 was considered significant.

Estimation of the invasiveness of serotypes and PFGE defined clones

In order to compare the probability of invasive disease due to individual serotypes and clones, empirical odds ratios (OR) and 95% confidence intervals ($CI_{95\%}$) were calculated by reference to all other serotypes and clones as previously described (5). The OR was calculated as follows: OR=(ad)/(bc), where *a* is the number of invasive A serotypes or clones, *b* is the number of carriage A serotypes or clones, *c* is the number of non-A serotypes or clones and *d* is the number of carriage non-A serotypes or clones. It follows from the presented formula that it is not possible to calculate an OR value when none of the isolates of a given serotype or clone is recovered from invasive infections or carriage. The choice of using all other serotypes and clones to measure the reference OR was substantiated by prior studies (reference 5 and references therein) that also provide a discussion of the strengths of this method.

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 2.1. 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 (figure 2.1), with seven isolates recovered from the 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 expressing these serotypes. The clones defined by PFGE are represented in figure 2.1. To further characterize the genetic lineages associated with each PFGE clone, selected isolates (n=75) were characterized by MLST. Six novel alleles (*adhP*58, *pheS*25 and -26, *atr*37, *glnA*36, *glcK*30) 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 separated into several PFGE clusters (Wallace index 0.411). However, one of the largest PFGE clusters (cluster C, Figure 2.1) 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 (SLVs) or double locus variants (DLVs)

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

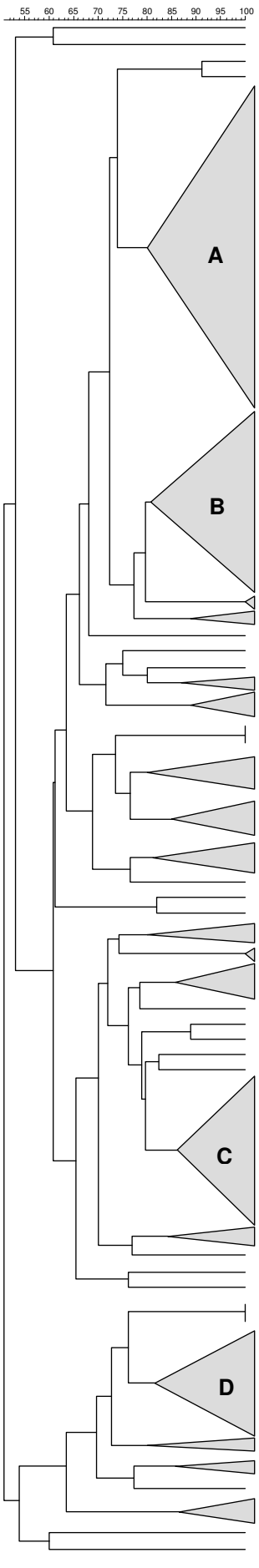
The OR were calculated for all serotypes identified among invasive isolates, and the results are presented in Table 2.1. The distribution of the capsular serotypes between EOD and LOD was not homogeneous. To test if the enhanced invasiveness of some serotypes correlates with an association with each 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 2.1.

TABLE 2.1 Enhanced disease potential of *Streptococcus agalactiae* serotypes

Serotype	Total (no.)	Colonization (no.)	Infection (no.)	OR ($CI_{95\%}$)[a]	EOD (no.)	OR ($CI_{95\%}$)	LOD (no.)	OR ($CI_{95\%}$)
Ia	60	42	18	2.11 (1.12-4.00)	13	2.42 (1.16-5.04)	5	NS
Ib	16	14	2	NS	1	NS	1	NS
II	54	46	8	NS	8	NS	0	NA
III	85	59	26	2.44 (1.37-4.34)	12	NS	14	6.23 (2.49-15.56)
IV	8	6	2	NS	1	NS	1	NS
V	66	59	7	NS	7	NS	0	NA
VII	5	5	0	NA	0	NA	0	NA
NT[b]	39	38	1	0.10 (0.01-0.72)	0	NA	1	NS
Total	333	269	64		42		22	

[a] An OR of >1 indicates increased invasive disease potential. NS, not significant; NA, not applicable.
[b] NT, non-typeable.

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 DLVs of each other and belong to the same eBURST group (figure 2.1). 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).



	Blood	CSF	Vaginal colonization
III	0	0	1
III	0	0	1
Ia	0	0	1
Ia	0	0	1
Ia	17 [ST23(5), ST24(4)]	1	40 [ST23(5), ST24(2), ST144(1)]
II	1 [ST24]	0	0
III	0	0	1
III	14 [ST17(3)]	5 [ST17(1)]	14 [ST17(2)]
NT	0	0	1 [ST17]
III	1	0	2
III	0	0	3 [ST17(1)]
V	0	0	1
II	0	0	1
II	0	0	1
II	0	1 [ST10]	2 [ST10(1)]
II	3	0	2 [ST10]
III	0	0	2 [ST290 (1)]
IV	0	0	1
V	0	0	2 [ST10(1)]
Ib	1 [ST8]	0	0
Ib	1 [ST10]	0	2
IV	0	0	2 [ST291(1)]
NT	0	0	1 [ST8]
II	1 [ST12]	0	1 [ST10]
Ib	0	0	4 [ST10(1)]
II	0	0	1
II	0	0	1
III	0	0	1
II	0	0	2 [ST12(1)]
III	0	0	1
III	0	0	3 [ST19(1)]
II	2 [ST28(1)]	0	4 [ST28(1)]
III	0	0	1
V	1 [ST289]	0	0
III	0	0	1
III	0	0	1
II	0	0	1
III	0	0	1
II	0	0	8 [ST28(3)]
III	2 [ST19, ST295]	0	18 [ST1(1), ST19(1), ST286(2)]
III	1	1 [ST19]	1
V	0	0	1
III	0	0	1
III	0	0	1
III	0	0	1
IV	0	0	1
V	0	0	1
IV	1 [ST2]	0	1
V	4 [ST1(1), ST2(2)]	0	13 [ST1(4), ST2(1)]
NT	0	0	1 [ST1]
V	0	0	3 [ST2(1)]
IV	0	0	1
V	2 [ST293(1)]	0	0
NT	1 [ST3]	0	0
III	1	1 [ST23]	3 [ST23(2)]
II	0	0	1
IV	1 [ST288]	0	0

FIGURE 2.1 PFGE, MLST, and sources of the isolates analyzed in this study. Shown is a dendrogram from PFGE SmaI macrorestriction profile analysis of 225 isolates from neonatal invasive infections and vaginal carriage in Portugal. Unweighted-pair group method using average linkages and the Dice coefficient (indicated as percentages in the scale above the dendrogram) were used to cluster the isolates. Each PFGE clone (defined as a group of ≥3 isolates with a Dice coefficient of ≥80%) is represented by a triangle with a size proportional to the number of isolates included in the cluster. The serotypes corresponding to the isolates are indicated, as well as the number of isolates recovered from blood, CSF or associated with vaginal colonization. In brackets are indicated the STs that were identified in each group, and the number in parenthesis following the ST indicates the number of isolates in which that particular ST was identified. The four major clones are identified by capital letters: A, serotype Ia, ST23 and ST24; B, serotype III, ST17; C, serotype II, ST28 and serotype III, ST19 and related STs; D, serotype V, ST1 and ST2.

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, $CI_{95\%}$ 1.95 to 10.98) and LOD (OR=10.59, $CI_{95\%}$ 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, $CI_{95\%}$ 1.08 to 4.62) but not for LOD (OR=2.05, $CI_{95\%}$ 0.80 to 5.23).

Discussion

Similarly 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 2.1 and figure 2.1). 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 (figure 2.1), indicating that all major lineages are capable of both asymptomatic colonization and causing invasive disease. The isolates belonging to each serotype were dispersed into 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 an increased invasive-disease potential. On the other hand, non-typeable 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, stratifying by early and late-onset infections, indicated that serotype Ia had a significant OR for EOD while serotype III was only significant 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, offer data regarding serotype prevalence in vaginal colonization, preventing an evaluation of the invasive potential 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 region, United Kingdom, 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), while in other cases a maternal source seems to be excluded due to negative vaginal and rectal colonization (24). 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 2.1). 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.

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CHAPTER 3

Group B streptococci causing neonatal infections in Barcelona are a stable clonal population: 18-year surveillance

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Summary

We analyzed 212 group B streptococci (GBS) from invasive infections in newborns in the Barcelona area between 1992-2009, with the aim of documenting changes in the prevalence of serotypes, antimicrobial resistance and genetic lineages and evaluating their association with either early-onset (EOD) or late-onset disease (LOD). Serotypes III (n=118) and Ia (n=47) together accounted for nearly 78% of the isolates. All isolates carried an alpha or alpha-like protein gene, with specific associations between genes and serotypes such as, Ib/*bca*, II/*bca*, III/*rib* and V/*alp3*, reflecting the presence of particular genetic lineages. Macrolide resistance (14.2%) was significantly associated to serotype V. Pulsed-field gel electrophoresis (PFGE) clustering was an excellent predictor of serotype and antibiotic resistance. The combination of PFGE and multilocus sequence typing revealed a large number of genetically distinct lineages. Still, specific lineages were dominant in our collection, particularly the serotype III/ST17/*rib* that had enhanced potential to cause LOD. Serotype Ia was concentrated on a single PFGE cluster composed of two genetic lineages: ST23/*eps* and ST24/*bca*. The ST24/*bca* sub-lineage of serotype Ia, that is infrequently found elsewhere, may be emerging as an important cause of neonatal invasive infections in the Mediterranean region. In spite of the introduction of prophylaxis, resulting in a pronounced decline of EOD, the study revealed a remarkably stable clonal structure of GBS causing neonatal infections in Barcelona in a period of 18 years.

Introduction

Streptococcus agalactiae, or group B streptococcus (GBS) is well established as a leading cause of neonatal sepsis and meningitis (25, 45). In neonates, early-onset disease (EOD) is defined as occurring within the first 7 days and late-onset disease (LOD) from day 8 to 90 (12). While vertical transmission is commonly accepted to be the cause of EOD (24, 37, 51), the source of bacterial strains causing LOD is less well understood (45). In 1996 guidelines for prevention of GBS neonatal infections by antimicrobial prophylaxis were published in the United States (12). Whereas a mixed risk-based and screening-based approach was initially suggested in the guidelines, the universal screening of pregnant women for GBS vaginal colonization at 35 to 37 weeks of gestation and the administration of intrapartum antimicrobial prophylaxis to carriers was proposed shortly afterwards (11). As a consequence, the incidence of EOD has fallen significantly over the past decade where these guidelines have been followed, yet this strategy is raising concern that the widespread use of intrapartum antimicrobials might delay, rather than prevent, the onset of GBS disease (10, 16). In the area of Barcelona, Spain, after the local implementation of the intrapartum antimicrobial prophylaxis guidelines, the incidence of EOD declined by 86%, from 1.92 cases per 1,000 live births in 1994 to 0.26 in 2001 ($p<0.001$) (2), and has remained at low levels since (ranging from 0.47 cases per 1,000 live births in 2007 to 0.18 in 2009). In the same area, the incidence of LOD increased from 0.11 cases per 1,000 live births in 1996 to 0.81 in 2009, but in spite of the difference between these values, these changes did not reflect a significant trend.

Furthermore, the increase in the use of antimicrobials due to intrapartum antibiotic prophylaxis can lead to the emergence of resistant bacteria (46), a concern that has been strengthened by the recent description of GBS strains showing reduced susceptibility to beta-lactams (28).

These considerations are driving the search for alternative prevention strategies. Studies evaluating the potential impact of vaccines in the management of GBS disease suggest that vaccination may provide additional benefits over antimicrobial prophylaxis, especially due to the expected reduction in LOD incidence (49). Vaccine formulations currently on trial are based on GBS capsular polysaccharides; however, they are not expected to provide optimal coverage in different regions, due to geographical differences in serotype distribution. In order to overcome serotype specificity, whole-

genome-based approaches have been directed toward identifying protein antigens, which hold promise as components of globally effective vaccines (26, 33).

In addition to the capsular polysaccharides, multiple virulence factors have been recognized and extensively characterized in recent decades. These virulence factors may be unevenly distributed within a particular serotype and may contribute significantly to the invasive potential of a particular lineage, independently of its capsular polysaccharide. Molecular epidemiology has been used to distinguish genetic lineages in order to probe for associations between specific GBS genotypes and disease. Most of these studies, using multilocus sequence typing (MLST), have identified a lineage with enhanced invasive capacity expressing serotype III and defined by sequence type 17 (ST17) (6, 15, 31). Moreover, in a study of carriage and invasive isolates from Portugal, we found that serotype Ia presented an enhanced invasive disease potential and that it was particularly associated with EOD (36). In that study, serotype Ia was associated mostly with a single pulsed-field gel electrophoresis (PFGE) cluster and with two sequence types (ST23 and ST24), again pointing to the possible existence of particular genetic lineages with enhanced invasive disease potential.

We undertook the analysis of GBS isolates responsible for invasive infections in newborns in the Barcelona area from 1992 to 2009 with the aim of documenting changes over this 18-year period and of testing associations with EOD and LOD. To this end, we have characterized the isolates with regard to their serotypes and antimicrobial resistance patterns and have identified the genetic lineages present by PFGE profiling, MLST, and surface protein gene profiling.

Materials and Methods

Eight hospitals located in the Barcelona metropolitan area monitored all EOD cases from 1994 on and all LOD cases from 1996 on. In these hospitals, GBS prevention policies were implemented progressively from 1994 onward. Invasive disease was defined as the presence of GBS in a normally sterile fluid (blood or cerebrospinal fluid [CSF]). During the period from 1994 to 2009, a total of 351.950 live infants were born in the 8 hospitals, and EOD was diagnosed in 243 cases (189 infants born in the 8 hospitals and 54 referred from other hospitals). During the period from 1996 to 2009, of a total of 315.576 live births, LOD was diagnosed in 131 infants.

Bacterial isolates

We characterized the 207 isolates available from the 374 GBS cases identified between 1994 and 2009 in the 8 Barcelona-area hospitals. Additionally, we also included 5 isolates recovered from patients with invasive GBS disease in the same centers between 1992 and 1993. Only the first isolate of each case was considered. A total of 212 GBS isolates were characterized: 123 from EOD and 89 from LOD patients.

Serotyping, antimicrobial susceptibility testing and macrolide-resistance phenotype

Capsular serotyping was carried out by a latex agglutination assay with a GBS serotyping kit (Essum, Umeå, Sweden) according to the manufacturer's instructions.

All GBS isolates were tested for susceptibility to erythromycin, clindamycin, tetracycline, chloramphenicol, levofloxacin, and penicillin by using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (13). The macrolide resistance phenotype was determined according to a double-disk test as described previously (38).

PCR determination of macrolide-resistance genotype

Total bacterial DNA was isolated by treatment of the cells with mutanolysin and boiling. Multiplex PCR was performed to detect the presence of the *erm*(B), *erm*(A) [*erm*(TR) subclass] and *mef* genes, as described elsewhere (17).

Pulsed-field gel profiling and MLST

Total bacterial DNAs of the strains were isolated, digested with SmaI, and separated by PFGE as described previously (36). Whenever a complete digestion with SmaI was not achieved, the isoschizomer Cfr9I was used (48). PFGE patterns were compared by using Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium) to create dendrograms by the unweighted pair-group method with arithmetic averages (UPGMA). The Dice similarity coefficient was used with optimization and position tolerance settings of 1.0 and 1.5, respectively. PFGE-based clusters were defined as isolates with ≥80% relatedness on the dendrogram (36). MLST was performed by sequencing seven housekeeping genes as described previously (27), and sequence types (STs) were identified by using the *S. agalactiae* MLST database (http://pubmlst.org/sagalactiae) and were analyzed using the entire database and goeBURST (20). Alleles and sequence types not previously described were deposited at the *S. agalactiae* MLST database. DNA sequences were analyzed by using Bionumerics software.

Surface protein gene profile

Total bacterial DNA was isolated by treatment of the cells with mutanolysin and boiling. A multiplex PCR assay was performed for direct identification of GBS alpha-like protein genes, as described elsewhere (14). This assay allowed the direct determination of the following GBS surface protein genes by analysis of the amplicon size: the alpha-C protein gene (*bca*); the epsilon protein gene (*eps*); and the *rib*, *alp*2/3, and *alp*4 genes. A previously described assay was performed to differentiate the *alp2* and *alp3* protein antigen genes (34).

Typing concordance and statistics

The Wallace (W) and adjusted Rand (AR) coefficients were calculated to determine the concordance between the different typing methods (9, 40, 47). The AR coefficient provides a measurement of the overall concordance between the results of two methods, whereas the W coefficient provides a directional measurement of clustering concordance between different typing methods, i.e., if the results of one typing method can predict the results of another method. Simpson's index of diversity (SID) was calculated to evaluate the diversity found among the isolates studied (9). All

these calculations were performed at the Comparing Partitions website (www.comparingpartitions.info). The Fisher exact test was used to evaluate associations. Odds ratios (OR) with 95% Wald confidence intervals ($CI_{95\%}$) (1), were calculated against all other serotypes or PFGE clusters and were used to identify particular serotypes or PFGE clusters associated with certain characteristics, controlling for a false discovery rate (FDR) under or equal to 0.05 (3). Spearman's non-parametric test was used to evaluate correlations (1).

Results

Capsular serotyping

The results of serotyping the 212 invasive GBS isolates from neonates are summarized in Table 3.1. Serotypes III (n=118) and Ia (n=47) were the most frequent among the population, together accounting for 77.8% of the isolates. The serotypes were also differently distributed in EOD and LOD (p=0.0067, Fisher's exact test). Serotypes III and Ia were found in 44% and 26% of EOD cases, respectively, and in 72% and 17% of LOD cases, respectively. In fact, the number of serotype III isolates found in LOD (n=64) was more than double the sum of all other serotypes (n=25), and this was the only serotype that showed a significant association with disease presentation (OR=2.980, $CI_{95\%}$ 1.581 to 5.734, for an association with LOD). Although more isolates were recovered from the CSF in LOD cases (n=25) than in EOD cases (n=21), in agreement with a previous report (36), we did not find any association between the serotype and the biological product from which the isolate was recovered.

TABLE 3.1 Serotype distribution among invasive GBS isolates causing EOD and LOD

Serotype	EOD			LOD			Total
	Blood	CSF	Total (%)	Blood	CSF	Total (%)	
Ia	27	5	32 (26)	11	4	15 (17)	47
Ib	8	0	8 (7)	4	1	5 (6)	13
II	9	1	10 (8)	1	0	1 (1)	11
III	41	13	54 (44)	46	18	64 (72)	118
IV	2	0	2 (2)	0	1	1 (1)	3
V	11	1	12 (10)	2	0	2 (2)	14
NT[a]	4	1	5 (4)	0	1	1 (1)	6
Total	102	21	123 (100)	64	25	89 (100)	212

[a]NT, nontypeable

PFGE cluster analysis and MLST

All isolates were analyzed by PFGE, and 43 different profiles were identified and were grouped into 18 different PFGE clusters (≥3 isolates), of which the major 5 accounted for nearly 60% of the isolates (Figure 3.1). The remaining isolates (n=34) were included in minor PFGE groups (≤2 isolates) or had unique profiles.

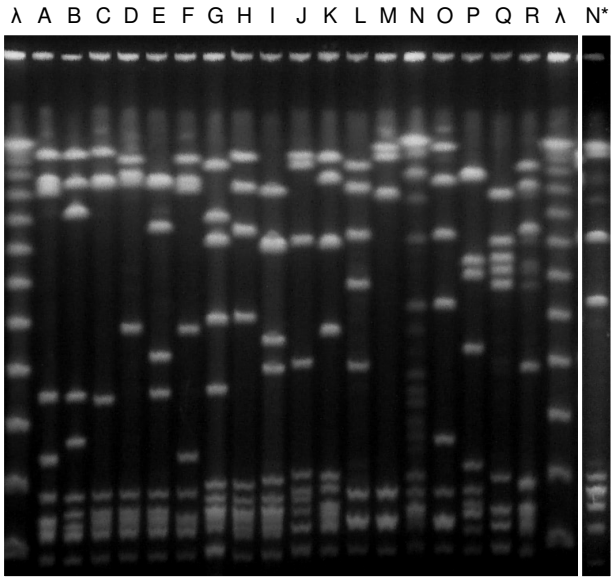

FIGURE 3.1 Representative SmaI macrorestriction profiles of each PFGE cluster. Capital letters above the lanes correspond to cluster designations. Lane N, incomplete digestion with SmaI; lane N*, the same strain as in lane N but now digested with Cfr9I (see the text); lane λ, Lambda Ladder PFG Marker (New England Biolabs, Beverly, MA).

The dendrogram depicting the relationship between these PFGE clusters is shown in Figure 3.2. The SID for the classification of the isolates into PFGE clusters was 0.894 ($CI_{95\%}$ 0.867 to 0.921), indicating that the collection analyzed is genetically very diverse. Each PFGE cluster was almost exclusively composed of isolates of the same serotype (W=0.955, $CI_{95\%}$ 0.910 to 1.000), indicating a very good predictive power of the PFGE-based genotypes for the serotype. The converse was not so; inspection of Figure 3.2 and Table 3.2 reveals that each serotype was subdivided into several PFGE clusters, as expected from the existence of several genetic lineages sharing the same serotype.

To further identify the genetic lineages associated with each PFGE clone, all isolates were characterized by MLST. Thirteen novel alleles and 13 novel STs (ST469 to ST471 and ST542 to ST551) were identified among the isolates studied. The detailed characterization of the clusters depicted in Figures 3.1 and 3.2 is summarized in Table 3.2.

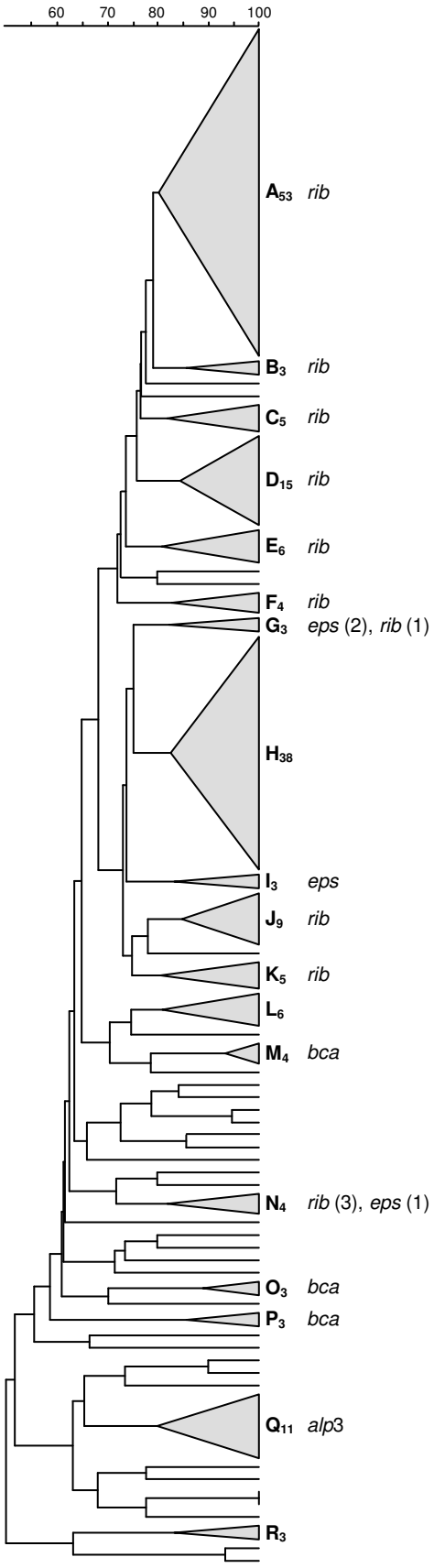

FIGURE 3.2 Dendrogram of the PFGE profiles of 212 GBS isolates and distribution of the surface protein genes. The dendrogram was constructed using UPGMA. Dice coefficients (percentages) are indicated in the scale above the dendrogram. Each cluster (defined as a group of three or more isolates with a Dice coefficient of ≥80%) is represented by a triangle proportional to the number of isolates included in the cluster. Clusters are designated by capital letters with a subscript number indicating the number of isolates included in the cluster. The surface protein genes found in each particular cluster are given on the right. If more than one gene was found in strains of the same PFGE cluster, the number of isolates carrying each gene is indicated in parentheses.

TABLE 3.2 Properties of the genetic lineages found among the 212 invasive isolates

PFGE cluster[a]	Serotype	EOD/LOD (no.)	STs in PFGE cluster with the same serotype (n)[b]	No. of isolates not susceptible to[c]			Susceptible isolates (no.)[e]
				ERY[d]	CHL	TET	
A_{53}	III	23/30	[ST17 (48), ST147 (1), ST148 (1), ST542 (1), ST543 (1), ST550 (1)]	1	0	51	2
B_3	III	2/1	[ST17 (2), ST180 (1)]	0	0	3	0
C_5	III	3/2	[ST17 (4), ST148 (1)]	0	0	5	0
D_{15}	III	3/12	ST17 (15)	0	0	15	0
E_6	III	2/4	[ST17 (4), ST469 (2)]	0	1	6	0
F_4	III	1/3	ST17 (4)	0	0	4	0
G_3	Ia	2/0	ST23 (2)	0	0	2	0
	III	1/0	ST19 (1)	1	1	1	0
H_{38}	Ia	22/14	[ST23 (25), ST24 (9), ST223 (1), ST545 (1)]	0	1	34	3
	III	0/2	ST17 (1), ST27 (1)	0	0	1	1
I_3	Ia	3/0	ST23 (3)	0	0	3	0
J_9	III	6/2	[ST19 (5), ST456 (1), ST471 (1), ST547 (1)]*	3[f]	0	6	1
	II	1/0	ST28 (1)*	0	0	1	0
K_5	III	0/3	ST17(3)	0	0	3	0
	NT[g]	1/1	ST28(2)	0	0	2	0
L_6	II	3/0	[ST2 (1), ST9 (1), ST12 (1)]*	1	0	0	2
	Ia	2/0	ST7 (2)*	2[h]	0	1	0
	Ib	0/1	ST1 (1)*	0	0	1	0
M_4	Ib	3/1	[ST8 (1), ST9 (2) ST10 (1)]	0	0	2	2
N_4	III	2/1	[ST19 (2), ST27 (1)]*	0	0	2	0
	V	1/0	ST19 (1)*	0	0	2	0
O_3	Ib	2/1	ST12 (3)	0	0	3	0
P_3	II	3/0	ST22 (3)	3[f]	0	3	0
Q_{11}	V	9/2	ST1 (11)	8	0	11	0
R_3	Ia	2/0	ST23 (2)	0	0	2	0
	NT[g]	1/0	ST19 (1)	0	0	0	1
$Other_{34}$	III	11/4	ST17 (4), ST19 (6), ST24 (1), ST106 (2), ST470 (1), ST546 (1)	6	3	14	1
	Ib	3/2	ST2 (1), ST9 (1), ST10 (1), ST12 (1), ST548 (1)	2	0	3	2
	II	3/1	ST1 (1), ST12 (1), ST22 (1), ST544 (1)	1[f]	0	3	1
	IV	2/1	ST2 (1), ST196 (1), ST549 (1)	0	0	2	1
	NT[g]	3/0	ST19 (1), ST27 (1), ST130 (1)	1[f]	0	1	1
	Ia	1/1	ST7 (1), ST196 (1)	0	0	1	1
	V	2/0	ST26 (1), ST551 (1)	1[f]	0	1	0
Total		123/89		30	6	189	19

[a]PFGE clusters are identified as indicated in Figure 3.1. Clusters are designated by capital letters and a subscript number indicating the number of isolates included in the cluster.
[b]Brackets indicate STs that were grouped into the same PFGE cluster and belonged to the same clonal complex by goeBURST and expressed the same serotype. Asterisks indicate STs or groups of STs that were found in the same PFGE cluster and belonged to the same clonal complex by goeBURST but expressed different serotypes.
[c]ERY, erythromycin; CLI, clindamycin; CHL, chloramphenicol; TET, tetracycline
[d]All isolates were simultaneously resistant to erythromycin and clindamycin, presenting with the cMLSB phenotype associated to the *erm*(B) gene, except when indicated.
[e]Isolates susceptible to all antimicrobials tested (ERY, CLI, CHL, and TET)
[f]One isolate carried the *erm*(A) gene
[g]NT, nontypeable
[h]Two isolates carried the *erm*(A) gene

The SID for the classification of the isolates according to their MLST-based sequence types was 0.805 ($CI_{95\%}$ 0.758 to 0.852), also indicating a genetically diverse collection but less diverse than the classification in PFGE profiles. Overall, the isolates sharing the same ST also shared the same serotype (W=0.978, $CI_{95\%}$ 0.961 to 0.997), indicating that MLST is a good predictor of serotype, a finding similar to that for the PFGE profiles.

In agreement with these observations, serotype III isolates representing ST17 were found mostly in PFGE clusters A_{53} and D_{15}. Likewise, most serotype Ia isolates (of which the majority belonged to ST23 but a significant proportion belonged to ST24) and serotype V isolates (ST1) grouped into particular clusters, H_{38} and Q_{11}, respectively.

Interestingly, most of the new STs were identified in serotype III isolates. Whereas ST469, ST542, ST543 and ST550 are single-locus variants (SLVs) of ST17, ST470 is a double-locus variant (DLV) of ST17 (exhibiting two novel alleles). On the other hand, ST471 and ST547 are SLVs of ST19, a sequence type previously associated with colonization (27, 31, 36). ST19 was found to be poorly represented in our collection; the largest PFGE cluster where this ST was found was J_9. This was expected, because the collection analyzed included only isolates that had caused invasive neonatal infections.

Six of the newly described STs (ST470, ST544, ST546, ST548, ST549, and ST551) were found in isolates included in minor PFGE clusters or showing unique profiles (Table 3.2). The combination of rare PFGE profiles and STs suggests that the GBS populations causing invasive neonatal disease may reflect the larger genetic diversity found in the overall GBS population to a limited extent.

In testing for an association between the largest PFGE clusters ($n \geq 10$) and the timing of disease presentation, both the major clusters of serotype III (A_{53} and D_{15}) presented significant OR for an association with LOD (OR=2.202, $CI_{95\%}$ 1.121 to 4.376 and OR= 6.182, $CI_{95\%}$ 1.598 to 35.220, respectively) but only the latter association was significant when controlled for the FDR (p=0.018). In agreement to what was found for the serotypes, none of these largest PFGE clusters was associated with recovery from blood or CSF.

Among sequence types, ST17 was the only genetic lineage defined by MLST that was overrepresented in LOD (OR=4.972, $CI_{95\%}$ 2.659 to 9.488). Of the two major serotype III/ST17 clusters defined by PFGE (A_{53} and D_{15}), only the latter was

significantly associated with LOD, suggesting that PFGE could distinguish between two ST17 lineages that are otherwise indistinguishable by their serotypes, ST and surface protein, but that could have other significant differences. This hypothesis remains to be studied further.

Antimicrobial susceptibility testing

All isolates were susceptible to penicillin and to levofloxacin. The overall rate of erythromycin resistance was 14.2% (n=30). All erythromycin-resistant isolates displayed the constitutive MLS_B phenotype ($cMLS_B$), defined by cross-resistance to all macrolides, lincosamides, and streptogramin B. None of the erythromycin-resistant isolates carried the *mef* gene,; the *erm*(B) gene was present in 76.7% (n=23) and the *erm*(A) [*erm*(TR) subclass] gene in 23.3% (n=7) of the isolates. Resistance to tetracycline was found in 89.2% (n=189) of the isolates and resistance to chloramphenicol in 2.8% (n=6).

While resistance to tetracycline or chloramphenicol was not clustered in particular serotypes, the same was not true for resistance to erythromycin ($p<0.001$, Fisher's exact test) (Table 3.2). Analysis of individual serotypes revealed that serotypes Ia (n=2/47 [4.3%]) and III (n=11/118 [9.3%]) presented less erythromycin resistance than expected (OR=0.219, $CI_{95\%}$ 0.024 to 0.926 and OR=0.408, $CI_{95\%}$ 0.165 to 0.962, respectively), while serotypes II (n=5/11 [45.5%]) and V (n=9/14 [64.3%]) presented more erythromycin-resistant isolates than expected (OR=5.788, $CI_{95\%}$ 1.297 to 24.7061 and OR=14.806, $CI_{95\%}$ 4.024 to 61.874, respectively), but only the finding for serotype V was significant when controlled for the FDR ($p<0.001$).

Analysis of the largest PFGE clusters ($n\geq10$) revealed three clusters with significant associations with erythromycin resistance, clusters H_{38} (OR=0, $CI_{95\%}$ 0 to 0.528) and A_{53} (OR=0.087, $CI_{95\%}$ 0.002 to 0.552) with reduced resistance and cluster Q_{11} (OR=21.092, $CI_{95\%}$ 4.645 to 131.992) with higher resistance; all these associations remained significant when controlled for the FDR ($p=0.009$ for both H_{38} and A_{53} and $p<0.001$ for Q_{11}). These results were not surprising. PFGE cluster Q_{11} includes the majority of serotype V isolates, all presenting with ST1, a sequence type that was also significantly associated with erythromycin resistance (OR=12.589, $CI_{95\%}$ 3.303 to 53.599). On the other hand, PFGE cluster H_{38} includes the majority of ST23/serotype Ia isolates, and cluster A_{53} represents the ST17/serotype III and related lineages. Both

clusters were associated with erythromycin susceptibility, a result that had already been suggested by the serotype analysis but that had failed to reach statistical significance. However, the MLST analysis revealed that both ST23 and ST17 were significantly associated to erythromycin susceptibility (OR= 0, $CI_{95\%}$ 0 to 0.658 and OR=0.040, $CI_{95\%}$ 0.001 to 0.256, respectively), supporting the indication given by the PFGE cluster analysis.

Even though ST19 was poorly represented in our collection, in agreement with its association with colonization (31), it was also overrepresented among erythromycin-resistant isolates (OR=6.885, $CI_{95\%}$ 2.082 to 22.557). This association was reported previously in Portugal (19).

Surface protein gene profiling

All isolates gave positive results for the presence of only one surface protein gene, with the exception of one isolate for which we failed to amplify any of the genes tested. The surface protein gene *rib* was the most prevalent, followed by the *eps*, *bca* and *alp*3 genes, showing variable distributions across serotypes (Table 3.3).

Table 3.3 Distribution of genes encoding surface proteins across serotypes

Serotype	Surface protein gene[a]				Total
	bca	***eps***	***rib***	***alp*3**	
Ia	12	**35**			
Ib	**11**	1		1	47
II	**7**	1	2	1	13
III	2		**116**		11
IV	1	2	1		118
V		2		**11**	3
NT[b]	1		5		13[c]
Total	34	41	123	13	6

[a]Boldface numbers indicate a significant correlation between surface protein gene and serotype (see the text)
[b]NT, nontypeable
[c]One isolate failed to amplify any of the surface proteins tested

No *alp*2 or *alp*4 genes were found among the isolates. There was a very high correspondence between serotypes and surface protein genes (AR=0.789, $CI_{95\%}$ 0.713 to 0.867). This was reflected in significant OR for the association of most serotypes ($n \geq 10$) with particular surface protein genes: serotype Ia with *eps*, serotypes Ib and II with *bca*,

serotype III with *rib*, and serotype V with *alp*3; all these association remained significant when controlled for the FDR ($p<0.001$) (Table 3.3).

Surface protein genes were differently distributed across PFGE clusters (Figure 3.2), correlating with the proportions of serotypes within the clusters. A main exception was serotype Ia isolates, which grouped in PFGE cluster H_{38}, although they presented with surface protein gene *eps* or *bca*. In this cluster, an absolute association was found between the ST and the surface protein gene, with all ST23 isolates carrying the *eps* gene and all ST24 isolates carrying the *bca* gene exclusively, in support of our hypothesis that they constitute sub-lineages. In addition, we have also identified two isolates, one representing ST223 and the other representing the newly identified ST545, that are SLVs of ST23 but not of ST24, both harboring the *eps* gene, suggesting diversification of ST23 with retention of the characteristic surface protein gene.

Discussion

This study comprises a considerable number of invasive GBS isolates collected over an 18-year period in the Barcelona region. In spite of the overall large number of isolates, the number of yearly infections was low, preventing detailed evaluation of the temporal changes in serotypes or PFGE clusters. Still, serotype III was present in all years and serotype Ia was absent in only two years of the study period. The remaining serotypes were represented by fewer isolates than the number of years studied, but there was an overall correlation between the number of isolates and the number of years in which they were found ($r= 0.964$, $p=0.0028$, Spearman's test), indicating that no significant changes in the serotypes causing neonatal infections occurred in Barcelona in the 18 years studied. A similar analysis by PFGE cluster is complicated by their larger number; still, each of the five largest clusters ($n\geq10$) was found in at least 7 years, and the two largest (A_{53} and H_{38}) were found in all but 2 of the study years. Taken together, these data reveal a remarkably stable clonal structure of the GBS causing neonatal infections in Barcelona over a period of 18 years. This occurred in spite of the major epidemiological changes in neonatal GBS infections due to the introduction of prophylaxis in 1994, which resulted in a pronounced decline in EOD incidence (2).

We found substantial diversity among the GBS isolates causing neonatal invasive disease, not only in terms of capsular polysaccharides, but also in the genetic lineages defined by both PFGE and MLST (Figure 3.2 and Table 3.2). However, most isolates belonged to two serotypes and to a few STs and major PFGE clusters. The serotype distribution found in the population was similar to that described in some European countries, where capsular types Ia and III prevail among isolates causing invasive neonatal infections (5, 36, 41). In contrast to some U.S. studies, where the prevalence of serotype V reached as high as 30% (42), and to the recent increase reported in the prevalence of this serotype in Scandinavia (4, 39), serotype V was found much less frequently in Barcelona, in agreement with the results of most studies across Europe.

Classification by PFGE and MLST defined groups of isolates frequently sharing the same serotype and surface protein gene, indicating that both techniques are identifying groups of closely related isolates. Strong correlations between the genes encoding surface proteins and serotypes were also found (Table 3.3): most serotypes were associated primarily with a single surface protein gene. The exception was

serotype Ia that was associated with two proteins, although only one reached significance, with approximately one-quarter of the isolates carrying the *bca* gene and the remaining three-quarters carrying the *eps* gene. While our data are consistent with those reported elsewhere in Europe, contrasting data can be found in some studies from the United States reporting the absence of the *bca* surface protein gene in all serotype Ia isolates causing invasive neonatal infections (32). We believe that this is a defining characteristic of a sub-lineage of serotype Ia, as discussed below. Also in contrast to the data reported here, another study from the United States found the *bca* surface protein gene in more than 50% of serotype V isolates (50). Considering that most studies describe a strong association of serotype V with the *alp*3 gene (21, 29, 43), it is possible that the higher prevalence of serotype V in the United States than in Europe results from the expansion of a sub-lineage not found in Europe. This is despite the fact that most serotype V isolates on either continent share the same sequence type (ST1).

As more collections are analyzed by their complement of surface protein genes, a broader understanding of these genes' relationships with serotypes may be obtained. However, our data suggest that it may be naïve to expect an absolute correlation with serotype and that additional typing methods, such as PFGE or MLST, are potentially useful for identifying distinct genetic lineages within each serotype that may also differ in their surface proteins.

Of note in this context was the high prevalence of ST24 found among serotype Ia isolates, which concentrated mostly in PFGE cluster H_{38}, together with the more widely disseminated ST23. ST24 is a DLV of ST23 that has rarely been found among large collections of GBS isolates characterized by MLST, with the exception of Portugal, where a significant prevalence of this ST was first reported in 2007 (36). Also in 2007, a study from Italy showed most serotype Ia isolates grouping together in the same PFGE cluster, presenting either the *bca* or the *eps* surface protein genes as described here, and the one representative isolate of this cluster characterized by MLST harbored the *bca* gene and was ST24 (21). Later, ST24 was found in 3 out of 52 serotype Ia invasive isolates from neonates in the United States; these 3 isolates were reported as a rare invasive clone (8). This suggests that within serotype Ia there are two different sub-lineages not distinguishable by PFGE, but discriminated by MLST and surface protein gene profiling. In agreement with this hypothesis is the absolute association between the ST and the surface protein gene in our collection, with all ST23

isolates carrying the *eps* gene and all ST24 isolates carrying the *bca* gene exclusively. Previous results from our laboratory had provided strong support for the circulation of both sub-lineages in Portugal (35). Taken together, these observations suggest that the presence of a particular alpha or alpha-like surface protein gene is a clonal property rather than a feature of the serotype. Interestingly, we have also found an additional ST24/*bca* isolate belonging to serotype III that may be the result of capsular switching (34).

This study, together with previous data from Portugal and Italy (21, 36) suggests that a particular sub-lineage of serotype Ia may be disseminated in the Mediterranean region. Although we could not find a correlation between each of the surface protein defined sub-lineages and the isolate source (blood or CSF) or the timing of disease presentation (EOD or LOD), the ST24 sub-lineage may have other properties that could explain its success.

In spite of the recent report of penicillin non-susceptibility among GBS (28) and the intensive use of beta-lactams in prophylaxis in Barcelona since 1994, all isolates were fully susceptible to penicillin. The macrolides can also be used in chemoprophylaxis, and in contrast to penicillin, a significant proportion of erythromycin resistance was found (14.2%), in line with previous results from a multicentre study in Spain (13.7%) (22). The phenotypes and genotypes of macrolide-resistant isolates from Barcelona were also similar to those previously identified in Spain (23). In agreement with other studies in different geographic regions, we found an association between macrolide resistance, serotype V (7, 53), and ST1 (44). Still, only 30% of erythromycin resistance was found in serotype V, while the remaining was dispersed in all other serotypes except serotype IV, a situation observed in a few previous studies (18, 30, 52). Resistance was also found to be dispersed in many genetic lineages as defined by PFGE and MLST, in agreement with the carriage of the methylase genes on mobile genetic elements. However, clonal expansion is also important in macrolide resistance; PFGE cluster Q_{11}, expressing serotype V and ST1, was found to be significantly associated with resistance. On the other hand, cluster A_{53}, representing the highly virulent ST17 and associated lineages, was less resistant than expected, reflecting the general observation that isolates representing ST17 are rarely macrolide resistant. The reasons why this highly virulent and successful lineage is seldom resistant are unknown.

The characterization of the population of GBS causing invasive infections in neonates in the Barcelona region revealed the existence of a large number of genetically distinct lineages that were present over a significant time span. The stability and dominance of a few lineages that are responsible for the majority of infections in spite of continuous antibiotic and immune selective pressures suggest that they are extremely well adapted to their particular niche. Although most of these lineages are widely disseminated worldwide, we have also identified seemingly regionally successful clones, raising the possibility of ongoing selection and expansion of specific virulent GBS clones. Continuous surveillance will shed further light on these processes and will determine if these clones will further expand beyond their current geographical boundaries.

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CHAPTER 4

Streptococcus agalactiae serotype Ib as an agent of meningitis in two adult non-pregnant women

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Summary

Two temporally and geographically clustered cases of meningitis caused by *Streptococcus agalactiae* expressing the infrequent Ib serotype are reported. Characterization by pulsed-field gel electrophoresis and multilocus sequence typing revealed that the isolates were identical and represented the widely distributed ST10/ST8 lineage associated with serotype Ib.

Case reports

Case 1

A 69-year-old woman, with no known risk factors for group B streptococcal (GBS) infection, was admitted on 7 January 2007 to a tertiary-care hospital in Lisbon with headache, nausea and vomiting, disturbances of consciousness, and blurred vision with an onset 24 hours prior to admission. On presentation, she was apyretic and confused and had no signs of meningeal irritation. Hematological investigations revealed a white blood cell (WBC) count of $4{,}630\times10^6$/liter (with 85% granulocytes) and a C-reactive protein level of 370 mg/liter. A lumbar puncture was performed, and blood samples were taken for culture. The cerebrospinal fluid (CSF) exhibited a WBC (granulocyte) count of 113/mm^3, a glucose concentration of 1mg/dl, a protein concentration of 533mg/dl, and Gram staining showed gram-positive cocci. A head computed tomography (CAT) scan was unremarkable.

On the basis of the initial results, empirical therapy consisting of ceftriaxone (4g daily, given intravenously) and ampicillin (12g daily, given intravenously) was started.

Clinical evolution was rapid with loss of consciousness and multiple organ failure, and the patient died 12h after admission.

Both CSF and blood cultures were positive 24h later for *Streptococcus agalactiae* susceptible to penicillin and ceftriaxone.

Case 2

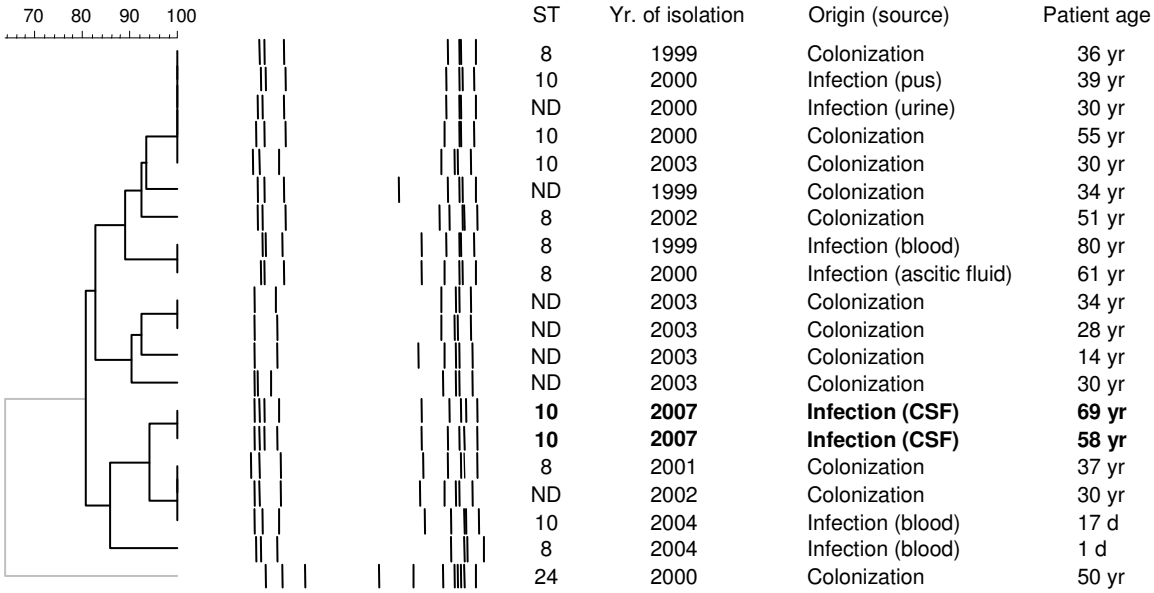
Within a few hours of the first case, a second case was encountered at the same hospital. A 58-year-old woman was admitted with headache, prostration, fever, polyarthralgia and diarrhea with an onset 48h prior to admission. On presentation, she was comatose and febrile, and had positive signs of meningeal irritation. The WBC count was $17{,}900\times10^6$/liter (with 88% granulocytes), and the C-reactive protein level was 292mg /liter. A lumbar puncture was performed, and blood samples were taken for culture. The CSF revealed >500WBC (granulocytes)/mm^3, a glucose concentration of <1mg/dl, a protein concentration of 736mg/dl, and the presence of gram-positive cocci. A head computed axial tomography scan was unremarkable.

On the basis of the initial results, empirical therapy consisting of ceftriaxone (4g daily, given intravenously) and ampicillin (12g daily, given intravenously) was started.

Both CSF and blood cultures were positive 24h later for *S. agalactiae* susceptible to penicillin and ceftriaxone. On the basis of the microbiological results, the antimicrobial therapy was changed to penicillin G (24×10^6 U daily, given intravenously).

Urine culture, a vaginorectal swab culture for detection of GBS, and another set of blood cultures performed 24h after admission were all negative. A review of the patient's clinical history revealed a mastectomy due to breast cancer 10 years prior to the current episode as the only possible risk factor for GBS infection. On discharge, the patient revealed no motor deficits but was diagnosed with neurosensory deafness and diplopia.

The patients lived on the same street, but interviews of the members of their households were not able to establish a prior social acquaintance. Isolates from both cases were serotyped by using specific sera (Denka Seiken, Japan) (5) and a genotyping method (9) as type Ib. Further characterization by pulsed-field gel electrophoresis (PFGE) (11) revealed identical profiles (Figure 4.1). To evaluate if the PFGE profiles were unusual among isolates expressing serotype Ib, 18 isolates from an ongoing nationwide survey of GBS infections focusing primarily on invasive infections (5, 11) were also characterized by both PFGE and multilocus sequence typing (8) and the results are reported in figure 4.1.



ST	Yr. of isolation	Origin (source)	Patient age
8	1999	Colonization	36 yr
10	2000	Infection (pus)	39 yr
ND	2000	Infection (urine)	30 yr
10	2000	Colonization	55 yr
10	2003	Colonization	30 yr
ND	1999	Colonization	34 yr
8	2002	Colonization	51 yr
8	1999	Infection (blood)	80 yr
8	2000	Infection (ascitic fluid)	61 yr
ND	2003	Colonization	34 yr
ND	2003	Colonization	28 yr
ND	2003	Colonization	14 yr
ND	2003	Colonization	30 yr
10	**2007**	**Infection (CSF)**	**69 yr**
10	**2007**	**Infection (CSF)**	**58 yr**
8	2001	Colonization	37 yr
ND	2002	Colonization	30 yr
10	2004	Infection (blood)	17 d
8	2004	Infection (blood)	1 d
24	2000	Colonization	50 yr

FIGURE 4.1 Characteristics of the GBS isolates responsible for the two cases of meningitis reported here (in bold) and of other serotype Ib isolates recovered in Portugal. The dendrogram was derived by the unweighted-pair group method using average linkages by using the Dice coefficient of the SmaI PFGE profiles of the isolates generated by using the Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium). The horizontal scale at the top represents the percentage of relatedness between isolates. ND, not determined. Patient ages are indicated in years (yr) or days (d).

Although the incidence of invasive GBS infections in non-pregnant adults has increased over the last decades, meningitis remains an uncommon manifestation (3). The incidence of GBS meningitis in adults in the United States is estimated to be 0.15 cases per 100,000 adults (2), but it results in a high case-fatality rate (27 to 34%) (3). The incidence in Portugal is not known, but a prior study on a large hospital of the Lisbon area did not identify any cases (5) and our ongoing nationwide survey started in 2003 identified only three cases, apart from the two reported here, among 916 GBS isolates responsible for infections in adults (136 of those were recovered from normally sterile sites).

The majority of GBS meningitis cases is closely linked to the presence of underlying conditions (2, 4) or to the perinatal period (6), but in some cases no patient risk factors could be identified (1). In some of these cases, evidence of an external source of infection was presented (7). In the two cases reported here, only the patient in the second case had a known risk factor for GBS invasive infection – a mastectomy following a breast cancer diagnosis. Farley *et al*. have suggest that a mastectomy may continue to enhance the risk for invasive GBS infection many years after it was

performed, but the presentation of the patients is cellulitis of the arm or chest wall on the side that underwent the mastectomy (4). This did not occur in our case, and in fact, even though sometimes a distant focus of infection can be established (2), this was not possible in either of the cases presented here. Moreover, in the second case, vaginorectal colonization could not be established, suggesting an exogenous source.

The temporal and geographical clustering of these two cases prompted further characterization of the isolates. The same serotype (Ib) was identified in both isolates. Serotype Ib is not among the most frequent serotypes associated with invasive disease in adults in the United States (3), and our previous study identified a single case of adult bacteremia due to this serotype (n=1/21) (5). Among neonatal infections and colonization of pregnant women in Portugal, serotype Ib was equally unremarkable in prevalence (11). Furthermore, none of the three adult GBS meningitis cases detected by our ongoing survey was associated with this serotype nor were any of the GBS infections in adults identified since January 2007 in the hospital where the two cases were detected (these isolates were serotyped as part of enhanced surveillance triggered by these meningitis cases).

Characterization by PFGE revealed identical profiles (Figure 4.1) indicating that the isolates responsible for these two cases belong to the same bacterial clone. The characterization by PFGE of the isolates in our collection as serotype Ib identified a major cluster, and although no other isolate revealed the same PFGE profile as the isolates responsible for the two cases described here, these were included in the same cluster as the majority of the Ib isolates. This cluster included isolates associated with colonization and responsible for different clinical presentations in diverse age groups (Figure 4.1). Multilocus sequence typing analysis confirmed the distinction of the isolates in two clusters, in agreement with the PFGE analysis, since two sequence types (STs), ST8 and ST10, are single locus variants and therefore closely related, whereas ST24 differs at five out of seven loci of both of them. Again, the isolates responsible for the two cases presented here had the same ST – ST10. This was not an unusual ST among Ib isolates and was found in four additional isolates associated with both colonization and infection, including invasive disease (Figure 4.1). ST8 and ST10 are also the most frequent STs found among serotype Ib isolates from various sources and geographic origins (8, 10). A previous study could not establish an association between a particular genetic lineage and invasive disease in adults, but a significant fraction of

infections caused by clonal complex CC9, where ST8 and ST10 are included, was noted (10).

Although a definite common source for the two cases was not established, their temporal clustering and the proximity of the homes of the two patients strongly suggested that such a common origin could exist. The microbiological findings further support this notion. Not only did the isolates present an unusual serotype, but they also displayed identical PFGE profiles, albeit belonging to the major genetic lineage associated with serotype Ib. The virulence of the strain causing these infections is apparent in the fatal outcome of one of the cases and the neurological sequelae of the surviving patient, in spite of appropriate antimicrobial therapy. GBS is an increasingly significant agent of bacterial meningitis in adults, and the high morbidity and mortality associated with these infections (3), even in adults with no risk factors, together with the possibility of community outbreaks, justify continued monitoring to obtain further insights into this important pathogen.

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CHAPTER 5

Dominance of a serotype Ia among group B streptococci causing invasive infections in non-pregnant adults in Portugal

E. R. Martins, J. Melo-Cristino, M. Ramirez, and the Portuguese Group for the Study of Streptococcal Infections. 2011. Dominance of serotype Ia among group B streptococci causing invasive infections in non-pregnant adults in Portugal. (Submitted to Journal of Clinical Microbiology)

Summary

The population of group B streptococcus (GBS) associated with invasive infections in non-pregnant adults in Portugal during 2001-2008 was analyzed (n=225). The isolates were characterized by pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), antimicrobial resistance and surface protein gene profiling. GBS invasive cases were found more frequently among men in all age groups. Whereas serotype V is dominant among invasive isolates recovered from non-pregnant adults elsewhere, serotype Ia was the most frequent in our collection that notwithstanding presented significant diversity. Serotype Ia was represented mainly by a single PFGE cluster defined by sequence types 23 (ST23) and surface protein gene *eps*; and ST24 and *bca*, as previously seen in neonatal invasive infections in Portugal, indicating that the same genetic lineages can be responsible for both vaginal colonization and invasive disease in all age groups. In contrast, the hypervirulent serotype III/ST17 neonatal lineage was responsible for a minority of infections. Serotype V isolates were distributed into two genetic lineages, one defined by ST1, surface protein gene *alp*3 and macrolide resistant, and another presenting with ST2, *eps* and fully susceptible to all antimicrobials tested. The *erm*(TR) gene was the most frequently found among erythromycin resistant isolates while the bovine associated *tet*(O) gene was found in a minority of tetracycline resistant isolates. Our data emphasizes the need for identifying the genetic lineages responsible for GBS invasive infections in non-pregnant adults. The dominance of serotype Ia in invasive disease in Portugal, regardless of age, highlights the importance of this serotype in GBS pathogenesis.

Introduction

Streptococcus agalactiae (group B streptococcus, GBS) is well established as a colonizing agent in pregnant women and an important cause of neonatal sepsis and meningitis (55). Nevertheless, in the past decade GBS has been increasingly associated with invasive disease in non-pregnant adults (23, 57). Such infections are held responsible for substantial morbidity and mortality, particularly in individuals with chronic underlying conditions such as malignancies, diabetes mellitus, cirrhosis and HIV infection, but several other risk factors have been identified in recent years (29). The spectrum of GBS disease in adults is broad, including more frequently bacteremia without a focus, skin and soft tissue, osteoarticular and urinary tract infections (23). Less frequent clinical presentations include meningitis and endocarditis that are, however associated with significantly higher morbidity and mortality (21, 40, 53). Although GBS infections are more frequently community acquired, nosocomial disease is also of concern (29).

With the introduction of antenatal screening guidelines and effective prophylaxis a significant decrease in neonatal invasive infections was observed in the last decade. On the contrary, the incidence of invasive infections unrelated to pregnancy in adults seems to be increasing worldwide (47, 54) justifying their increased study. Since the 1990's, serotype V emerged in the US as the most frequent GBS serotype causing invasive disease in non-pregnant adults (33, 57). Later, other serotypes such as Ia and III have also been recognized worldwide as significant causes of invasive disease (23, 45, 62), with the exception of Japan, where serotype Ib is reported as the leading cause of invasive disease in adults (44).

GBS isolates still remain mostly susceptible to penicillin, the first drug of choice for the prophylaxis and treatment of GBS disease. However, increased levels of erythromycin and clindamycin resistance are being described worldwide, raising concerns as to their use as alternative second-line agents (5, 12, 27). Intrapartum antibiotic prophylaxis is proposed to contribute to the emergence and dissemination of antibiotic-resistant clones (13), as would be also expected from the high antibiotic usage among the older age groups. Therefore, the need for new preventive strategies for GBS disease is now focusing on the development of vaccines. While the first studies targeted exclusively the polysaccharide capsule, its poor immunogenicity and variable prevalence in different countries led to the suggestion of exploring vaccination

approaches that could overcome serotype-specificity. Polysaccharide-protein conjugate vaccines, showing more favourable immune responses than polysaccharide only vaccines, have undergone clinical trials with promising, although still preliminary, results (2, 22). Currently, purely protein vaccines, based on components of the recently described GBS pilus-like structures, are being discussed in the literature (10, 38).

DNA-based typing methods such as pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) are now extensively used in molecular typing of GBS isolates, alongside classical serotyping and antimicrobial susceptibility testing. These methods have been helpful in discriminating GBS populations into specific genetic lineages, some recognized for their enhanced virulence potential (6, 28, 36, 42). Nevertheless, the diversity of genotype-phenotype combinations found among GBS isolates suggests ongoing diversification of GBS genetic lineages, to which capsular transformation is likely contributing, perhaps not as frequently as initially thought (41). Therefore, continuous surveillance and epidemiological studies are needed to ensure an accurate knowledge of how GBS serotypes and proteins are distributed among clones, across countries and over time, as this will impact on the vaccine strategies under development.

The aim of our study was to characterize the isolates recovered from invasive disease in non-pregnant adults in Portugal, to evaluate how serotype, antimicrobial resistance and surface protein genes are distributed among PFGE and MLST-based genetic lineages, and to compare them to those responsible for neonatal invasive infections.

Materials and Methods

Bacterial isolates

A collection of 225 GBS isolates recovered from 2001 to 2008 from invasive disease in non-pregnant adults (≥ 18 years old) in Portugal was analyzed. The isolates were provided by the Microbiology laboratories of 24 Portuguese hospitals, which were asked to submit all non-duplicate GBS isolates recovered from normally sterile sites. Isolates and data were collected as part of a laboratory-based surveillance program but no audits of reporting laboratories were performed to ensure compliance.

Submitted isolates included those recovered from blood (n=183), ascitic fluid (n=15), synovial fluid (n=14), cerebrospinal fluid (CSF) (n=7), and pleural effusion (n=6). Isolates were identified to the species level by Gram stain, colony morphology, catalase test, and a commercial latex agglutination technique (Slidex Strepto B, bioMérieux, Marcy L'Étoile, France).

Serotyping

Capsular serotyping of all isolates was carried out by a latex agglutination assay with a GBS serotyping kit (Essum, Umeå, Sweden) according to the manufacturer's instructions.

Antimicrobial susceptibility testing and macrolide-resistance phenotype

Susceptibility testing for penicillin G, erythromycin, clindamycin, tetracycline, chloramphenicol and levofloxacin was performed by disk diffusion. Clinical and Laboratory Standards Institute (CLSI) methods and interpretation criteria were used for all antimicrobial agents (14). The macrolide resistance phenotype was determined according to a double-disk test with erythromycin and clindamycin as previously described (43).

Macrolide-resistance genotypes and tetracycline-resistance determinants

Total bacterial DNA was isolated by treatment of the cells with mutanolysin and boiling. The presence of the *erm*(B), *erm*(A) [*erm*(TR) subclass] and *mef* genes was detected by a multiplex PCR reaction, as described elsewhere (24). Two isolates that were erythromycin resistant and did not amplify the *erm*(B), *erm*(A) nor the *mef* genes were also tested for the presence of the *erm*(T) gene (59). To further discriminate *mef*

genes into *mef*(A) or *mef*(E) an additional PCR was performed (56). All tetracycline resistant isolates were screened for the presence of the *tet*(K), *tet*(L), *tet*(M) and *tet*(O) genes as previously described (58).

Pulsed-field gel profiling and MLST

PFGE was performed to all isolates as previously described (42). Briefly, chromosomal DNA of the strains was prepared and digested with SmaI or, whenever necessary due to incomplete digestion, its isoschizomer Cfr9I, and separated by PFGE. The Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium) was used to generate a dendrogram with the comparison of the PFGE patterns by the unweighted pair-group method with arithmetic averages (UPGMA). The Dice similarity coefficient was used with optimization and position tolerance settings of 1.0 and 1.5%, respectively. PFGE-based clusters were defined as groups of 3 or more isolates with ≥80% relatedness on the dendrogram (42). MLST was performed by sequencing seven housekeeping genes as described previously (31), and sequence type (ST) assignment was done by using the *S. agalactiae* MLST database (http://pubmlst.org/sagalactiae) and analyzed using the entire database and goeBURST (26). Alleles and sequence types not previously described were deposited at the *S. agalactiae* MLST database. Analysis of DNA sequences was done by using Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium).

Surface protein gene profile

Total bacterial DNA was isolated by treatment of the cells with mutanolysin and boiling. A multiplex PCR assay was performed for direct identification of GBS alpha-protein-like genes, as described elsewhere (15). The GBS alpha-C protein gene (*bca*) and the epsilon (*eps*), *rib*, *alp*2/3, and *alp*4 surface protein genes were identified by direct analysis of the amplicon size. The *alp2* and *alp3* protein antigen genes were differentiated by a previously described assay (41).

Typing concordance and statistics

Wallace coefficient (W) provides a quantitative measure of the clustering concordance between different typing methods (11, 48). In our collection, the Wallace coefficient was calculated to determine the concordance between PFGE-based

clustering, serotyping and surface protein gene profiling. Simpson's index of diversity (SID) was used to evaluate the diversity found among the isolates studied (11). Both these calculations as well as the 95% Confidence Intervals ($CI_{95\%}$) were performed with the web tools available at www.comparingpartitions.info. Information about the sex ratios in different age groups in the Portuguese population were obtained from Statistics Portugal (www.ine.pt). The Fisher exact test was used to evaluate associations. The odds ratio (OR) with 95% Wald confidence intervals (CIs) (1), were calculated against all other serotypes or PFGE clusters and used to identify particular serotypes or PFGE clusters associated with certain characteristics, controlling for the false discovery rate (FDR) under or equal to 0.05 (3).

Results

Isolates

The collection of 225 GBS invasive isolates was recovered from patients with ages ranging from 19 to 92 years old, with a mean age of 63 years. Two sub-populations were considered in this study: the younger sub-population, defined as patients ranging in age from 18-64 years old, and the elderly sub-population (≥ 65 years old). Overall, the GBS isolates were more frequently recovered from men (n=132, 58.7%) than from women (n=93, 41.3%) but this varied markedly according to the age group (Table 5.1).

TABLE 5.1 Distribution of the 225 GBS isolates by sex and age groups

Sex	Age group (%)				Total	
	18-64 years		≥65 years			
M	64	(28.4)	68	(30.2)	132	(58.7)
F	31	(13.8)	62	(27.6)	93	(41.3)
Total	95	(42.2)	130	(57.8)	225	(100.0)

Also, the majority of GBS isolates were recovered from the elderly and accounted for nearly 58% of all isolates. Whereas in the younger sub-population the number of isolates recovered from men (n=64) more than doubled the number recovered from women (n=31), in the elderly sub-population the isolates were recovered equally from both men and women (n=68 and n=62, respectively). To test if there was an overrepresentation of men, we made to simplifying assumptions: 1) there was no sex or age bias on reporting; and 2) the entire Portuguese population could be considered at risk of developing GBS invasive infection. According to the Portuguese demographic data available at Statistics Portugal (www.ine.pt), in the period of 2001 to 2008 the mean sex ratio in Portugal among adults (15-64 years old) was 0.97 males/female and 0.72 males/female in the elderly (≥65 years old). In our collection of GBS isolates, the mean sex ratio of invasive infections among the adults and the elderly were 2.1 and 1.1 males/female, respectively. Considering the Statistics Portugal sex ratios, there is a 2-fold increase in invasive disease among younger men as compared to the number of expected cases in a 0.97 males/female ratio, and a 1.5-fold increase in invasive disease among elderly men as compared to the number of expected cases in a

0.72 males/female ratio, suggesting that men are always at increased risk of having GBS invasive infection independently of age.

Serotype and MLST distribution among PFGE clusters

The results of serotyping are summarized in Table 5.2. Overall, serotype Ia was considerably more prevalent and nearly 9% of the isolates (n=19) were non-typeable with considerable overall serotype diversity (SID=0.794, CI95% 0.765 to 0.822). To our knowledge this is the first time a serotype VI isolate is identified in Portugal. This serotype is known to be one of the most prevalent among colonizing serotypes pregnant women in Japan and Malaysia (34), but was rarely found elsewhere.

Again, serotypes were differently distributed according to patient age groups (Table 5.2).

TABLE 5.2 Serotype distribution among age groups

Serotype	Age group (%)				Total	
	18-64 years		≥65 years			
Ia	42	(18.6)	36	(16.0)	78	(34.7)
Ib	8	(3.6)	12	(5.3)	20	(8.9)
II	11	(4.9)	15	(6.7)	26	(11.6)
III	14	(6.2)	20	(8.9)	34	(15.1)
IV	1	(0.4)	1	(0.4)	2	(0.9)
V	14	(6.2)	30	(13.3)	44	(19.6)
VI	0		1	(0.4)	1	(0.4)
VII	0		1	(0.4)	1	(0.4)
NT	5	(2.2)	14	(6.2)	19	(8.4)
Total	95	(42.2)	130	(57.8)	225	(100.0)

In the younger sub-population, serotype Ia was isolated three times more frequently than other serotypes such as III, V and II, while in the elderly sub-population, although serotype Ia was still the most prevalent, serotypes such as V and III were almost as frequent. We then tested if there was a different distribution of each serotype in the two sub-populations. Although serotype Ia reached significance in the younger sub-population (Fisher's exact test, p=0.011), it did not remain significant after FDR correction, indicating that, in spite of the differences described, none of the serotypes is statistically significant in their different distribution between the two age groups considered.

Serotype II was found to be more frequently isolated from synovial fluid than expected from its representation in invasive isolates (Fisher's exact test, p=0.01). No other significant associations were found between the biological source of the isolate and either patient characteristics (sex or age), or any of the bacterial features studied such as serotype or antimicrobial resistance.

PFGE analysis revealed 33 different profiles defined at ≥80% similarity that grouped in 13 PFGE clusters (having three or more isolates), of which the major five accounted for nearly 70% of the overall collection (Figure 5.1). The remaining isolates (n=26) were included in minor PFGE clusters (containing two or fewer isolates) or had unique profiles (Figure 5.1). The SID for the classification of the isolates in PFGE clusters was 0.858 ($CI_{95\%}$ 0.824 to 0.893), indicating that the collection analyzed is very diverse. The concordance between PFGE-based genotypes and serotype as given by $W_{PFGE \rightarrow serotype}$=0.730 ($CI_{95\%}$ 0.638 to 0.821), showed that 73% of any pair of isolates in the same PFGE cluster also share the same serotype.

Further analysis of the genetic lineages associated with each PFGE cluster included the characterization by MLST of representative isolates of all serotypes (n=81, corresponding to at least 30% of the isolates of each serotype). Among the isolates studied we identified five novel alleles [*adhP*(87), *pheS*(35), *glnA*(49), and *sdhA*(43 and 44)] as well as five novel STs (ST472 to ST474, ST497 and ST498).

The detailed characterization of the clusters depicted in Figure 5.1 is summarized in Table 5.3. While most serotype Ia isolates grouped together in a particular cluster (C_{73}), serotype III isolates distributed mostly into two different clusters (A_{15} and D_{15}), and serotype V isolates were mainly found in three clusters (K_{19}, L_{10} and M_5). Serotype Ia isolates presented mainly with ST23 and ST24 in agreement with similar observations in neonatal invasive disease and colonization in pregnant women (39, 42).

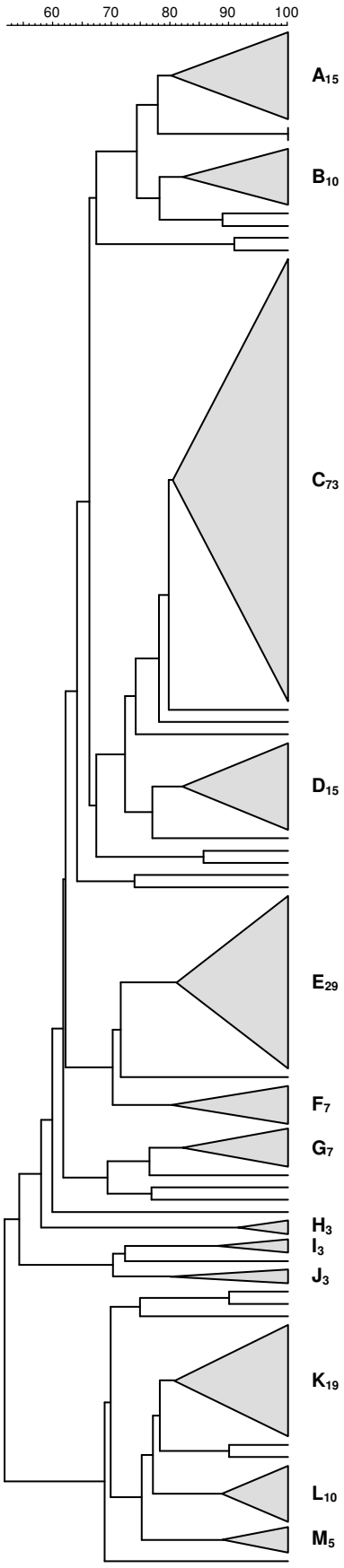

FIGURE 5.1 Dendrogram of the PFGE profiles of 225 GBS isolates. The dendrogram was constructed using the unweighted pair group method with arithmetic mean (UPGMA) method. Dice coefficients (percentages) are indicated in the scale above the dendrogram. Each cluster (defined as a group of three or more isolates with a Dice coefficient ≥80%) is represented by a triangle proportional to the number of isolates included in the cluster. Clusters are designated by capital letters and a subscript number indicating the number of isolates included in the cluster.

TABLE 5.3 Distribution of serotypes, MLST-based sequence types and antimicrobial susceptibility profiles among PFGE clones

PFGE cluster[a]	Serotype (no.)	STs in PFGE cluster (no.)[b]	Macrolide resistance phenotype genotype (no.)			Tetracycline resistance genotype (no.)
			$cMLS_B$	$iMLS_B$	M	
A_{15}	III (11)	[ST10 (1), ST19 (1), ST286 (1)]*, ST498 (1)	0	*erm*TR (3)	0	*tet*M (9)
	II (2)	ST28 (1)*	0	0	0	*tet*M (2)
	V (2)	ST19 (2)*	0	0	0	*tet*M (2)
B_{10}	II (7)	ST28 (1)*	0	0	1[c]	*tet*M (6)
	V (2)	ST19 (1)*	0	0	0	*tet*M (2)
	NT (1)	ST28 (1)	0	0	0	*tet*M (1)
C_{73}	Ia (66)	[ST23 (13), ST24 (6), ST144 (1)]*	0	0	*mef*E (2)	*tet*M (64)
	NT (3)	None	0	0	0	*tet*M (3)
	II (2)	ST28 (1)	0	0	0	*tet*M (2)
	Ib (1)	ST23 (1)*	0	0	0	*tet*M (1)
	III (1)	ST17 (1)	0	0	0	*tet*M (1)
D_{15}	III (13)	ST17 (4)	*erm*B (1)	0	0	*tet*M (10), *tet*O (1)
	NT (2)	None	0	0	0	*tet*M (2)
E_{29}	Ia (7)	ST8 (5)*, ST23 (2)	*erm*B (1), *erm*TR (1)	0	0	*tet*M (7)
	Ib (15)	[ST8 (2), ST9 (1), ST10 (3), ST12 (1)]*	*erm*TR (1)	*erm*TR (1)	0	*tet*M (13)
	V (5)	ST10 (1)*	*erm*B (2)	0	0	*tet*M (4)
	II (1)	ST12 (1)*	*erm*TR (1)	0	0	*tet*M (1)
	NT (1)	None	0	0	0	*tet*M (1)
F_7	II (4)	ST474 (1)*	0	0	0	*tet*M (2)
	Ib (2)	ST10 (1)*	0	0	0	0
	NT (1)	ST10 (1)	0	0	0	0
G_7	NT (3)	ST12 (1)*	0	0	0	*tet*O (3)
	II (2)	ST12 (1)*	*erm*TR (1)	0	0	*tet*O (2)
	V (2)	ST12 (1)*	0	0	0	*tet*O (2)
H_3	NT (3)	ST130 (1)	1[c]	0	0	0
I_3	Ia (1)	ST88 (1)*	0	0	0	0
	Ib (1)	None	0	0	0	0
	II (1)	ST88 (1)*	0	0	0	0
J_3	III (2)	ST23 (1)*	0	0	0	*tet*M (1), *tet*O (1)
	Ia (1)	ST23 (1)*	0	0	0	0
K_{19}	V (13)	[ST1 (4), ST497 (1)]*	*erm*B (1), *erm*TR (1)	*erm*TR (5)	0	*tet*M (12)
	NT (5)	ST1 (1)	0	0	0	*tet*M (5)
	VII (1)	ST1 (1)*	0	0	0	*tet*M (1)
L_{10}	V (10)	ST2 (3)	0	0	0	0
M_5	V (5)	ST1 (2)	*erm*TR (1)	*erm*TR (2)	0	*tet*M (5)
$Other_{26}$	II (7)	[ST28 (1), ST472 (1)]*	0	0	0	*tet*M (4), *tet*O (1)
	III (7)	None	*erm*B (2)	0	0	*tet*M (2)
	V (5)	None	0	0	0	*tet*M (2), *tet*O (1)
	Ia (3)	None	0	0	*mef*E (1)	*tet*M (2)
	IV (2)	[ST196 (1), ST473 (1)]*	0	0	0	*tet*M (2)
	Ib (1)	None	0	0	0	*tet*M (1)
	VI (1)	ST10 (1)*	0	0	0	0
Total	225		14	11	4	181

[a] PFGE clusters are presented as indicated in Figure 5.1. Clusters are designated by capital letters and a subscript number indicating the number of isolates included in the cluster.
[b] Brackets indicate STs that were grouped into the same PFGE cluster and belonged to the same clonal complex by goeBURST and expressed the same serotype. Asterisks indicate STs that were grouped into the same PFGE cluster and belonged to the same clonal complex by goeBURST but expressed different serotypes.
[c] Isolates failing to amplify the *erm*(B), *erm*(TR), *mef*(A/E) and *erm*(T) genes.

Interestingly, a small number of serotype Ia isolates (n=7) grouped in a different cluster (E_{29}), together with other isolates exhibiting mainly serotype Ib (n=15). These serotype Ia isolates presented mostly with ST8 (n=5), a sequence type that was previously found in Portugal among invasive and colonizing GBS isolates presenting exclusively the type Ib capsular serotype (40).

Serotype V was the second most prevalent serotype in our collection. While the isolates in clusters K_{19} and M_5 presented with ST1, suggesting the existence of two sub-lineages within this sequence type distinguishable only by PFGE, the isolates found in cluster L_{10} were ST2. These observations suggest the existence of two main lineages presenting this serotype. Serotype III isolates were predominantly represented in clusters A_{15} and D_{15}, the first comprising mostly ST19, previously associated to colonization (31, 36), and the later accounting for the "hyper-virulent" ST17 lineage (37, 50). However, this lineage is poorly represented in our collection when compared to its overrepresentation in neonatal invasive disease (42).

Surface protein gene profiling

All except two isolates were positive for the presence of only one surface protein gene that were diversely distributed in the bacterial population (SID=0.747, $CI_{95\%}$ 0.727 to 0.768). The surface protein gene *bca* was the most prevalent, followed by the *eps*, *rib*, *alp*3 and *alp*2 genes, showing variable distributions across serotypes (Table 5.4).

TABLE 5.4 Distribution of genes encoding surface proteins across serotypes

Serotype	Surface protein gene[a]					Total[b]
	bca	***eps***	***rib***	***alp*2**	***alp*3**	
Ia	25	**47**	2	2	2	78
Ib	**15**	2	1	2		20
II	7	1	**17**	1		26
III	3		**29**	2		34
IV		2				2
V	7	15	2		**18**	42
VI	1					1
VII					1	1
NT[b]	8	3	4		4	19
Total	66	70	55	7	25	223

[a] Highlighted in bold whenever a significant correlation between surface protein gene and serotype was found (see text)
[b] Total number of isolates excludes two serotype V isolates which failed to amplify any surface protein gene

The *alp*4 gene was not found among the isolates. There was a significant association of most serotypes with a particular surface protein gene, namely Ia and *eps*; Ib and *bca*; II and *rib*; III and *rib*; and V and *alp*3 (all Fisher's exact test $p<0.0001$). Nevertheless, the concordance between PFGE-based genotypes and surface protein genes was low $W_{PFGE \rightarrow surface\ protein}=0.566$ ($CI_{95\%}$ 0.491 to 0.641), as was the concordance between serotype and surface protein genes, $W_{serotype \rightarrow surface\ protein}=0.466$ ($CI_{95\%}$ 0.409 to 0.523). These results reflect the fact that some serotypes frequently present with more than one surface protein gene, as seen for serotype Ia isolates that are mainly associated to surface protein genes *eps* and *bca*, and for serotype V that presents with *alp*3 and *eps*, although only one of these genes reached significance in each serotype (Table 5.4). Furthermore, serotype Ia isolates are mainly grouped together in a particular PFGE cluster (C_{73}), regardless of presenting with surface protein genes *eps* or *bca*, thus having a significant contribution for a lower Wallace coefficient.

Antimicrobial susceptibility testing

The frequency of resistance to the antimicrobials tested differed among serotypes and was unevenly found in the PFGE clusters (Table 5.3). All isolates were susceptible to penicillin and chloramphenicol, 1.3% (n=3) were resistant to levofloxacin and 80.4% (n=181) to tetracycline.

The overall rate of erythromycin resistance was 12.9% (n=29), higher than that documented previously (10.7%) in a Portuguese study analysing the GBS isolates responsible for invasive and non-invasive disease as well as colonization (24). Among the erythromycin-resistant isolates, 14 displayed the constitutive Macrolide-Lincosamide-Streptogramin B resistance phenotype ($cMLS_B$), 11 the inducible phenotype ($iMLS_B$) and 4 the M phenotype. All erythromycin resistant isolates were tested by PCR for the presence of genes responsible for the macrolide resistance. Two isolates (one presenting with the M and the other with the cMLSB phenotypes) failed to amplify any of the genes *erm*(B), *erm*(A) [*erm*(TR) subclass] or *mef*, and were further tested for the presence of the *erm*(T) gene, frequently found among *Streptococcus bovis* isolates but already detected in GBS isolates (19), and again there was no amplification. Among the remaining 27 PCR-positive isolates, the *erm*(B) gene was present in 25.9% (n=7), the *erm*(TR) gene in 63.0% (n=17), and the *mef* gene in 11.1% (n=3) of the isolates. All $iMLS_B$ isolates carried the *erm*(TR) gene, whereas the $cMLS_B$ presented

with either *erm*(B) or *erm*(TR). Such an association between $iMLS_B$ and *erm*(TR) was previously reported in Canada (17). The three isolates presenting the M phenotype carried the *mef*(E) variant. Even though the MLS_B phenotype was found to be distributed across serotypes and not particularly clustered; the M phenotype (in the *mef*-positive isolates) was found exclusively in serotype Ia isolates. Erythromycin resistance was more frequent among serotype V isolates, in agreement with previous observations in other countries (12, 18, 60, 61); however, this association was not significant after FDR correction. In an analysis of individual clusters, erythromycin resistance was found to be overrepresented in cluster K_{19} (OR=7.453, $CI_{95\%}$ 1.783 to 66.65), which clustered together most isolates of serotype V presenting with ST1, and underrepresented in serotype Ia cluster C_{73} (OR=0.207, $CI_{95\%}$ 0.066 to 0.689), both significant after FDR correction (p=0.021 and p=0.044, respectively). Also, the high concordance between PFGE-based genotypes and erythromycin resistance (Ery^R) as given by $W_{PFGE \rightarrow EryR}$=0.873 ($CI_{95\%}$ 0.815 to 0.931), indicates that PFGE clustering is a good predictor of erythromycin resistance. For instance, despite the fact that cluster E_{29} grouped together isolates presenting four different serotypes, all of these serotypes included at least one macrolide resistant isolate.

Among the tetracycline resistant isolates, 94% (n=170/181) carried the *tet*(M) gene and 6% (n=11/181) the *tet*(O) gene. The *tet*(K) and *tet*(L) genes were not detected in any of the isolates tested. Tetracycline resistance was found in all major PFGE clusters with the exception of L_{10}, a cluster of serotype V isolates presenting with ST2. Interestingly, the majority of the isolates carrying the *tet*(O) gene grouped together in the same PFGE cluster (G_7), regardless of the different capsular types expressed by the isolates included in the cluster (Table 5.3).

Discussion

This is the first study exclusively focused on the analysis of the invasive group B streptococcal infections in non-pregnant adults in Portugal. The main limitation of our study is the fact that this is not a population-based study, although our network does include the majority of hospital-based microbiology laboratories. Taken together with the lack of audits to the reporting laboratories to ensure compliance, this makes it likely that not all cases of GBS invasive disease occurring in Portugal are reported within our surveillance network. Accordingly, we have refrained from calculating the incidence of GBS invasive disease in Portugal, since we would probably underestimate the actual incidence. Despite these limitations, we believe the collection studied is large enough to reflect the bacterial population diversity and to identify the major clones causing invasive disease among adults in Portugal. Also, since it is laboratory based surveillance, it is unlikely that any sex or age bias exists when reporting GBS invasive cases, indicating that any sex or age imbalance in the collection reflects the actual characteristics of the GBS invasive infection cases. Another limitation of our study is the absence of clinical data, which prevents us from evaluating the associations between GBS infection and patient history, including underlying medical conditions that were previously shown to be risk factors for GBS invasive infection (29).

GBS invasive infections were more frequent in the elderly, reflecting the possible impact of risk factors that increase with age, such as co-morbidities and immune senescence. GBS invasive infections were also substantially more frequent in men, regardless of age. Our findings are in agreement with a higher proportion of invasive infections among younger and elderly men reported elsewhere (35, 57), but the reasons behind the higher susceptibility of men remain to be clarified. It was suggested previously that sex could be a surrogate marker for other risk factors including underlying medical conditions (29), however our study was not designed to address this issue.

The GBS isolates characterized here revealed substantial diversity of genetic lineages defined by both PFGE and MLST (Figure 5.1 and Table 5.3), similar to that reported among isolates causing neonatal invasive infections in both Portugal (42) and Barcelona (39). However, the collection characterized here did present higher diversity in terms of surface protein genes and serotype than a similar size collection of invasive isolates from neonates (39), with higher SID values and non-overlapping $CI_{95\%}$. In

adults we also found GBS isolates of serotypes VI and VII and isolates carrying the *alp*2 gene for the first time as agents of infection in our country. Taken together, these observations suggest a more diverse genetic structure of the GBS population causing invasive disease in adults when compared to neonates. A possible explanation for this difference could be the multiplicity of clinical presentations of invasive infections in adults, together with frequent and varied underlying medical conditions presented by these patients. A previous history of antimicrobial consumption is another factor that may increase the diversity of the GBS population by selecting for resistant isolates.

A very conserved and consistent serotype distribution is observed among the GBS isolates causing neonatal invasive disease in Europe, including Portugal, and the US, where capsular types Ia and III are preponderant (4, 42, 49). However, in the GBS isolates causing invasive disease in non-pregnant adults in Portugal, we observed that the dominant serotypes contrast with those found in other countries. Our data shows that serotype Ia was significantly more prevalent (35%), followed by serotypes V (20%), III (15%), II (12%), and Ib (9%). In other countries such as Spain (8), Sweden (46), Norway (4), the US (57), Australia and New Zealand (62), serotype V is the leading cause of invasive infections in non-pregnant adults, including the elderly, and serotype Ia is much less frequent. It is possible that in Portugal there are specific lineages of serotype Ia that are well adapted to their particular niche and that may be expanding. We have previously described the increased invasive potential of serotype Ia lineages presenting with ST23 and ST24, mainly when causing early-onset disease (EOD) in neonates (42). Considering the similar clonal structure observed in this study, it is possible that the same lineages are particularly prone to cause invasive infections in adults. Taken together, the dominance of serotype Ia in invasive disease in adults, as well as the previously documented importance of this serotype in neonatal invasive infections as well as colonization in pregnant women (39, 42), it is clear that the same genetic lineages can be responsible for both colonization and invasive disease in all age groups. We also found a small number of ST8 serotype Ia isolates grouping in a different cluster (E_{29}), together with other isolates exhibiting mainly serotype Ib but also other serotypes, and presenting with ST8 and its single-locus variants (SLVs) ST9, ST10 and ST12. Moreover, these STs share only two alleles (in the *atr* and *glc*K loci) with ST24 and none with ST23, indicating that this lineage of serotype Ia isolates is unrelated to the dominant ST23 and ST24 lineage and may have resulted from capsular

switching. The recipient of this putative capsular switching event would belong to the lineage defined by PFGE cluster E29, as most of these serotype Ia and Ib isolates are not only grouped into the same PFGE cluster but also share the same surface protein (*bca*) and closely related STs. This particular lineage was not previously found among GBS serotype Ia isolates in Portugal characterized in previous studies, but had already been recovered from invasive and colonizing isolates in adults presenting exclusively the type Ib capsular serotype (40). This observation is consistent with this new serotype-genotype combination having arisen from capsular switching and possibly having an adaptive advantage for causing invasive infections in adults, explaining why the Ia/ST8/*bca* lineage was not previously associated with vaginal colonization or neonatal infections.

Serotype V was the second most prevalent serotype, distributed in three PFGE clusters, and characterized by different sequence types and surface protein genes. While the isolates in cluster K_{19} and M_5 presented with ST1, the surface protein gene *alp*3, and a significant proportion of macrolide resistance, the isolates found in cluster L_{10} were ST2, displayed the surface protein gene *eps* and were fully susceptible to all antimicrobials tested, including tetracycline. The existence of this lineage justifies that serotype V was the only serotype significantly associated to tetracycline susceptibility (Fisher's exact test P=0.0002).

The prevalence of serotype III was much lower than that found in neonatal invasive disease in Portugal (42). The two main clusters of serotype III isolates, A_{15} and D_{15}, presented mainly with ST19 and ST17, respectively. Whereas ST19 was previously associated to colonization, ST17 is recognized as a major lineage responsible for neonatal invasive infections (31, 36). The underrepresentation of this lineage in our collection, in agreement similar observations elsewhere (37), re-enforces the importance of other serotypes, particularly serotype Ia, in invasive infection in adults.

We found a strong correlation between the genes encoding surface proteins and serotypes (Table 5.4). However, the correspondence is not absolute and the surface protein gene proved helpful in discriminating different genetic lineages within serotypes. Similarly to what was reported previously (39), serotype Ia was associated to two surface protein genes, *bca* and *eps*, although only *eps* reached significance. Considering the high number of *bca* carrying isolates in our collection, and the perfect association of this surface protein gene with ST24 (39), this particular sub-lineage

seems equally adept at causing infections in adults as it is in neonates. Future studies, particularly in other European countries, should help evaluate if serotype Ia is expanding in the continent or if, on the contrary, this represents successful clones within the more limited geographical boundaries of the Mediterranean region. Nevertheless, previous reports of the ST24 sub-lineage as a rare clone in both Europe (52) and the US (7) may indicate ongoing changes in the clonal composition of GBS causing invasive infections worldwide.

Two distinct surface protein genes, *alp*3 and *eps*, were found to be similarly distributed among serotype V isolates, even though only the first was significantly associated to this serotype, mainly because *eps* is also overrepresented in the ST23/serotype Ia isolates. Considering that most studies describe a strong association of serotype V with the *alp*3 gene (27, 32, 51), it is possible that our observation of a similar frequency of the genetic lineage V/ST2/*eps* reflects the expansion of a sub-lineage not frequent elsewhere.

When comparing our surface protein gene profiling results to those found among neonatal invasive isolates (39), a considerable difference was also found in serotype II isolates. Even though the number of serotype II isolates causing invasive disease in neonates was smaller than in adults, the isolates responsible for neonatal infections carried mainly the *bca* gene, while in this study the number of isolates carrying the *rib* gene more than doubled those carrying the *bca* gene. In addition, we found that the isolates carrying the *bca* gene presented mostly with ST12 (and its double-locus variant ST474) whereas the isolates carrying the *rib* gene presented mainly with ST28 (and its SLV ST472). Both ST472 and ST474 were newly identified in this study and suggest diversification of two different serotype II lineages carrying different surface protein genes. It is possible that the *rib* carrying lineage of serotype II isolates shows a higher propensity to cause joint infections (4 out of the 5 serotype II isolates recovered from synovial fluid presented with the surface protein *rib*), being responsible for the overall association of this serotype with synovial fluid.

Erythromycin resistance was found to be similarly distributed among the constitutive and inducible MLS_B phenotypes ($cMLS_B$, n=14; and $iMLS_B$ n=11), while in a previous study in Portugal the $cMLS_B$ phenotype was three times more prevalent than the $iMLS_B$ (24). The number of isolates carrying the *erm*(TR) gene more than doubled the number of isolates carrying the *erm*(B) gene, also the opposite of what had been

previously reported in the country (24). This could be due to the expansion or disappearance of specific clones carrying these genetic elements or to the fact that in the previous study both invasive and non-invasive isolates were characterized (24). In contrast to the data reported here, several publications indicate that the *erm*(B) gene is overrepresented in serotype V isolates (9, 25, 27, 30, 61), while a higher prevalence of *erm*(TR) has only been described in Canada (17). This suggests that there are several distinct erythromycin resistant genetic lineages expressing serotype V that are differentially distributed in various geographic locations. The fact that macrolide resistance was significantly associated to specific PFGE-based clusters but not to particular serotypes suggests that increasing resistance is due in part to the limited expansion of resistant clones, although horizontal dissemination of genetic elements carrying resistance determinants may have also contributed. Considering the wide consensus on the importance of serotype V in erythromycin resistance, the fact that this serotype is overrepresented in almost all studies of GBS causing invasive infections in non-pregnant adults, and the diversity of resistant lineages, a detailed discrimination of the macrolide resistant clones from other regions may help understand the evolution and dynamics of erythromycin resistance in GBS.

The nearly ubiquitous resistance to tetracycline was mostly mediated by the *tet*(M) gene, as described elsewhere (52). A small fraction of the isolates carried the *tet*(O) gene, which has been associated with bovine strains (20). Not all erythromycin resistant isolates were simultaneously resistant to tetracycline, including those carrying the *erm*(B) gene, indicating that erythromycin resistance is not necessarily linked to tetracycline resistance and may not be encoded by the same mobile genetic elements, as previously suggested (16).

Even though the *tet*(M) gene is spread throughout all serotypes and major PFGE clusters, the association between the *tet*(O) gene and a particular genetic lineage, as defined by the same PFGE cluster (G_7) and sequence type (ST12), supports the clonal expansion of this resistance determinant.

GBS is a significant agent of invasive disease in adults, and the high morbidity and mortality associated with these infections justifies the continued monitoring of this pathogen. While most serotypes and genetic lineages are capable of both asymptomatic colonization and of causing invasive disease in different age groups, their prevalence is not the same, suggesting that particular lineages may be better adapted to specific

lifestyles or age groups. On the other hand, the salient role played by serotype Ia isolates as cause of invasive infections in both adults and neonates in the Iberian Peninsula, together with the high prevalence of ST24 that is rarely found elsewhere, suggests that lineages with enhanced invasiveness may be emerging at a regional level. This dynamic nature of GBS invasive isolates may prove challenging to future prevention strategies for these infections.

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CHAPTER 6

Evidence for rare capsular switching in *Streptococcus agalactiae*

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Summary

The polysaccharide capsule is a major antigenic factor in *Streptococcus agalactiae* (Lancefield group B streptococcus – GBS). Previous observations suggest that exchange of capsular loci is likely to occur rather frequently in GBS, even though GBS is not known to be naturally transformable. We sought to identify and characterize putative capsular switching events, by means of a combination of phenotypic and genotypic methods, including pulsed-field gel electrophoretic profiling, multilocus sequence typing, and surface protein and pilus gene profiling. We show that capsular switching by horizontal gene transfer is not as frequent as previously suggested. Serotyping errors may be the main reason behind the overestimation of capsule switching, since phenotypic techniques are prone to errors of interpretation. The identified putative capsular transformants involved the acquisition of the entire capsular locus and were not restricted to the serotype-specific central genes, the previously suggested main mechanism underlying capsular switching. Our data, while questioning the frequency of capsular switching, provide clear evidence for *in vivo* capsular transformation in *S. agalactiae*, which may be of critical importance in planning future vaccination strategies against this pathogen.

Introduction

Streptococcus agalactiae (group B streptococcus, GBS) is primarily a colonizing agent of the genitourinary and gastrointestinal tracts, but it is also a leading cause of bacterial sepsis and meningitis in neonates and is increasingly associated with invasive infections in adults (39). The capsular polysaccharide is a major GBS virulence factor and also the main target of antibody-mediated killing (11). In the last decade, conjugated multivalent vaccines have been developed and proved to be highly immunogenic, raising the possibility of the prevention of perinatal GBS disease through maternal immunization (38).

Nine capsular types are recognized: Ia, Ib and II to VIII, along a new provisional serotype IX, recently proposed (19). Comparison of the capsular locus genes suggested that the structural diversity of the capsular polysaccharide is associated with the genetic diversity of the capsular locus, possibly driven by horizontal gene transfer (9, 24). Capsular serotyping has been the classical method used in epidemiological studies to differentiate GBS isolates, although further characterization of GBS diversity includes the use of a broad range of DNA-based typing methods, such as restriction fragment length polymorphisms (RFLP), pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). Both PFGE and MLST have provided new clues about the population structure of *S. agalactiae*, particularly the recognition of diverse lineages among serotype III that were shown to differ in virulence potential and tropism (16, 25, 26, 31, 41). Although the distinction of lineages within a particular serotype has proved useful, a complete correlation between capsular type and the lineages defined by MLST was not found (4, 21, 22). Moreover, whole-genome comparative analysis of isolates expressing different serotypes showed that they sometimes share more genes than strains of the same serotype, suggesting a serotype-independent clustering of strains (43). These observations support the hypothesis that closely and divergently related clones may share the genes coding for a particular capsular type, suggesting that exchange of capsular genes *in vivo* may have occurred (16, 21, 22). We refer to these phenomena as capsular switching *in vivo*, recognizable by the expression of different serotypes and the presence of different capsular loci in otherwise undistinguishable isolates when sampling a set of 11 loci distributed in the genome.

The changes at the capsular locus were proposed to be driven by the equilibrium between the selective pressure imposed by host immunity and conservation of a

particular capsular polysaccharide, as an adaptive advantage of virulent clones (4, 9, 21). Capsular switching by homologous recombination would be facilitated by the organization of the locus encoding the capsular polysaccharide synthesis genes (*cps*), where the highly variable serotype determining region (*cps*G-*cps*K) is flanked by conserved genes (9, 24). This led to the suggestion that genetic exchange of the central part of the *cps* operon could be driving capsular switching (9, 22). According to Luan *et al.*, who specifically addressed this issue, horizontal transfer of capsular genes occurs at a high level within a population without restriction to genetic background. The authors of that study also suggest that since only advantageous combinations of genotype-serotype persist, these altered serotypes, due to capsular switching, are recognized at a lower frequency among stable clones (21).

Capsular switching is well established in other streptococcal species such as *Streptococcus pneumoniae,* where spontaneous *in vivo* capsular transformation events were observed and characterized (28, 34). In contrast to GBS, *S. pneumoniae* is naturally transformable, and this is widely believed to be responsible for the ease with which this species exchanges DNA. Capsular switching may have serious impact in pneumococcal vaccination programs since it may provide the selective pressure for virulent genotypes to switch capsules and escape vaccine coverage (6), and a similar response could be seen with a future introduction of GBS vaccination (38).

The aim of the present study was to evaluate the concordance between serotype and the clusters defined by PFGE and to further characterize any putative transformants to establish unequivocally that capsular switching occurs in GBS. We combined PFGE with the analysis of multiple genes spread across the GBS genome in order to identify capsular transformants and concluded that capsular switching events occur less frequently than previously thought.

Materials and Methods

Bacterial isolates

We studied a collection of 463 GBS isolates isolated between December 1999 and December 2004 in 11 Portuguese hospitals. The isolates were recovered from neonates (invasive infections – defined by the isolation of GBS from a normally sterile fluid) and adults (invasive and non-invasive infections and asymptomatic colonization), some of which were previously characterized (12, 23, 25). Isolates were identified to the species level by Gram stain, colony morphology, catalase test, and the commercial latex agglutination technique Slidex Strepto B (bioMérieux, Marcy l'Etoile, France).

Serotyping

Capsular serotyping was done by slide agglutination using sera for types Ia, Ib and II to VIII (Hemolytic Streptococcus Typing antisera for Group B; Seiken, Japan), as previously described (12). Further confirmation of serotyping results was carried out by a latex agglutination assay with a GBS serotyping kit (Essum, Umeå, Sweden) according to the manufacturer's instructions or by the capillary precipitation method (40) with type II, III, and V sera (Statens Serum Institute, Copenhagen, Denmark).

Pulsed-field gel profiling and MLST

Total bacterial DNA of the strains was isolated, digested with SmaI, and separated by PFGE as previously described. PFGE patterns were compared by using Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium) to create dendrograms by the unweighted pair-group method with arithmetic averages (UPGMA). The Dice similarity coefficient was used with optimization and position tolerance settings of 1.0 and 1.5, respectively. PFGE-based clusters were defined as isolates with ≥80% relatedness on the dendrogram (25).

MLST was performed by sequencing seven housekeeping genes as described previously (16) and sequence type (ST) identification was done by using the *S. agalactiae* MLST database (http://pubmlst.org/sagalactiae). Alleles and sequence types not previously described were deposited at the *S. agalactiae* MLST database. Clonal complex analysis was done using the entire GBS MLST database and goeBURST (13).

Surface protein gene profile

Total bacterial DNA was isolated by treatment of the cells with mutanolysin and boiling. A multiplex PCR assay was performed for direct identification of GBS alpha-protein-like genes, as described elsewhere (10). This assay allowed the determination of the following GBS surface protein genes directly by the analysis of the amplicon size: the alpha-C protein gene (*bca*); the epsilon protein gene (*eps*); and *rib*, *alp*2/3 and *alp*4 genes. Later, we used the sequences of the surface protein antigen genes *alp*2 and *alp*3 deposited at Genbank to set up a similar multiplex PCR assay to differentiate these two genes by direct evaluation of the amplicon size. Oligonucleotide primers, their target sites and sequences are listed in Table 6.1. For detection of the β antigen gene (*bac*) a PCR assay was performed as described previously (27).

TABLE 6.1 Oligonucleotide primers designed in this study

Primer	Target		Sequence (5'→3')	Amplicon size (bp)[b]
	Gene	Accession no. (position)[a]		
alp2/3-D	*alp*2 *alp*3	AF208158 (1070) AF245663 (1311)	CATTAACCGTCACTCCAGAGCAAC	
alp2-R	*alp*2	AF208158 (1582)	CTTCATCTGTTGACTTATCTGGATAG	513*
alp3-R	*alp*3	AF245663 (1637)	CTTTTGGTTCGTTGCTATCCTTAAG	327*
PI1-UP	*gbs*80	AE009948 (632375)	GGTCGTCGATGCTCTGGATTC	881
PI1-DN	*gbs*80	AE009948 (633255)	GTTGCCCAGTAACAGCTTCTCC	881
PI2a-UP	*gbs*67	AE009948 (1417645)	CTATGACACTAATGGTAGAAC	575
PI2a-DN	*gbs*67	AE009948 (1417071)	CACCTGCAATAGACATCATAG	575
PI2b-UP	*san*_1519	AAJR01000022 (10160)	ACACGACTATGCCTCCTCATG	721
PI2b-DN	*san*_1519	AAJR01000022 (10880)	TCTCCTACTGGAATAATGACAG	721
PI1_all-UP	*sal*_0710	AAJP01000027 (23718)	ACCTATGTTGCTGATTCGGCTGAAAATG	684†
PI1_all-DN	*sal*_0709	AAJP01000027 (23035)	TACGGACACTTTCTAGTGCCTTTGGATC	684†
neuB1-UP	*neu*B	EF990364 (10162)	CAAGCGGTGAATATTTTACG	2797
neuA-DN	*neu*A	EF990364 (12958)	CATTGCTTCCTTTATATGCCATG	
neuB2-UP	*neu*B	EF990364 (10653)	CTTGGGACAAGAAGCGCAAG	
neuC1-UP	*neu*C	EF990364 (11153)	GCGTTGATTTATAATGTCCCAG	
neuC2-UP	*neu*C	EF990364 (11672)	CTGATTGGTAATTCGTCTTCTGG	
neuD-UP	*neu*D	EF990364 (12133)	GAAGATGGCTCAATAGATGCAG	

[a] That is, the position of primer from the 5' end

[b] *, together with primer alp2/3-D; †, size if the isolate lacks the PI-1 islet (if this locus is present, a 16.7 Kb-fragment would be expected).

Pilus-associated gene profile

The genes encoding pili in GBS are located within two distinct loci in different regions of the genome, designated pilus islands 1 and 2 (PI-1 and PI-2), the later presenting two distinct variants, PI-2a and PI-2b (35). We designed a multiplex PCR

targeting the sortase genes to identify the pilus islands present in each isolate by direct evaluation of the amplification product size. Oligonucleotide primers, their target sites, and sequences are listed in Table 6.1. Briefly, 1 µl of DNA template lysate prepared as described above was added to the PCR mixture containing 1× PCR buffer (Promega, Madison, WI), 200 µM deoxynucleoside triphosphates (MBI Fermentas, Vilnius, Lithuania), 0.5 µM primers, 1.5 mM $MgCl_2$, and 1 U of GoTaq DNA polymerase (Promega) in a final volume of 50 µl. PCR conditions for amplification were as follows: 30 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min in a Biometra T-gradient (Goettingen, Germany) thermocycler. The PCR products were analyzed by electrophoresis in a 2% (wt/vol) agarose gel.

In order to confirm that the PI-1 negative isolates did not carry the pilus pathogenicity islet or parts of it, a set of primers that flanked the PI-1 locus were designed with the aim of amplifying the entire pilus islet. Oligonucleotide primers, their target sites, and sequences are listed in Table 6.1. All isolates that were negative for the presence of the PI-1 sortase gene were subjected to PCR with primers PI1_all-UP and PI1_all-DN. If the pilus locus was absent, the expected PCR product using these primers would be 684 bp. The PCR mixture and PCR conditions were similar to those described above except for the annealing temperature that was 60ºC.

Capsule gene cluster RFLP analysis

Sequence variability within a region of the *cps* gene cluster (*cps*G-*neu*A) results in different patterns between serotypes upon enzyme digestion, allowing the unambiguous identification of the *cps* gene clusters. We performed a previously described PCR-based restriction fragment length polymorphism (RFLP) method suitable for genetic serotyping of *S. agalactiae* (24) to identify which *cps* cluster was present in each isolate.

Southern-blot hybridization

Chromosomal DNA fragments digested by SmaI and separated by PFGE, were transferred to nylon membranes (Hybond N^+; Amersham, Uppsala, Sweden) by using the vacuum gene system (Pharmacia LKB Biotech, Uppsala, Sweden) and membranes were hybridized to specific DNA probes labeled with the ECL Direct Labeling System

(Amersham, Uppsala, Sweden). Both hybridization and labeling were performed according to the manufacturer's instructions.

Ia, Ib, and II to V serotype-specific DNA probes were generated by PCR with template DNA from reference strains, as previously described (3), and hybridized against all isolates tested. The PCR products were purified by using the High Pure PCR Product Purification Kit (Roche, Manheim, Germany) and labeled according to the manufacturer's instructions.

Sequencing of the *cps* locus

Sequence analysis of the partial GBS *cps* gene cluster, namely, the conserved regions *cps*D-*cps*G and *neu*B-*neu*A, reveal polymorphisms that allow the identification of the various *cps* loci. A previously published method described a GBS serotype identification method by PCR and sequencing of the *cps*D-*cps*G region. In this region 56 variable sites suitable for molecular serotyping were identified (18). For the *neu*B-*neu*A region, we used a similar PCR and sequencing analysis approach. The oligonucleotide primers designed for the amplification and sequencing of this region are listed in Table 6.1. We aligned the sequences obtained from at least three isolates expressing serotypes Ia, Ib, and II to V in order to identify the variable sites characteristic of each serotype. Later, we analyzed the data obtained by sequencing of the *neu*B-*neu*A region of the isolates being tested for concordance with these polymorphisms. Both these methods allow the confirmation of the conventional capsular serotyping method and also the evaluation of the possibility of partial *cps* gene cluster transfer and recombination among isolates of different serotypes.

Typing concordance and DNA sequence analysis

Wallace coefficient (W) provides a quantitative measure of the clustering concordance between different typing methods (7, 30). We assessed the Wallace coefficient in our collection to determine the concordance between PFGE-based clustering and serotyping. Simpson's index of diversity (SID) was calculated to evaluate the diversity found among the isolates studied (8). These calculations were performed at the comparing partitions website (www.comparingpartitions.info).

Analysis of DNA sequences was done using the Vector NTI 10 (Invitrogen, Carlsbad, CA) and the GATA (29) software.

Nucleotide sequence accession number

The sequence of the *cps*G-*cps*H region of strain 312754 was deposited in Genbank under accession GQ457335.

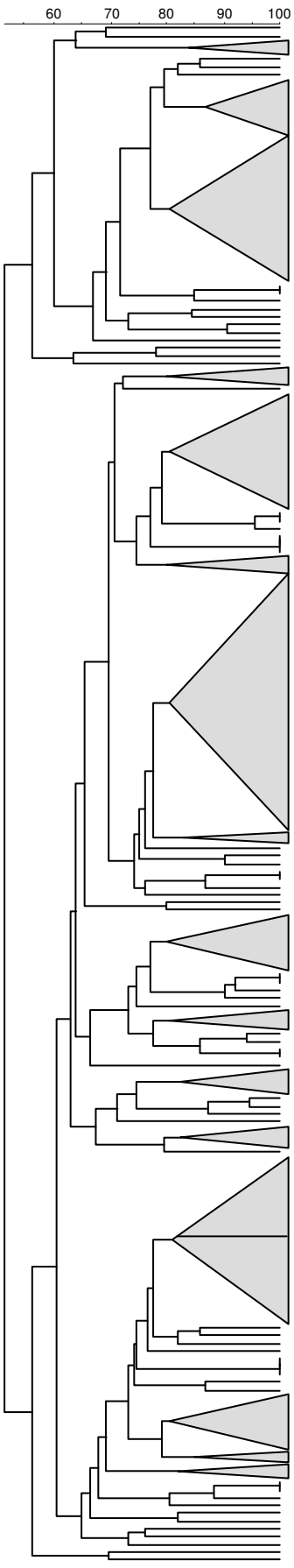
Results

Serotyping and PFGE

Among the 463 isolates we found 16 different PFGE clusters (≥ 5 isolates), of which the major five accounted for nearly 60% of the isolates (Figure 6.1). The remaining isolates (n=67) were included in minor PFGE groups (containing four or fewer isolates) or had unique profiles and were not considered for further analysis. The SID for the classification of the isolates in PFGE clusters was 0.901 (95% confidence interval, $CI_{95\%}$ 0.887 to 0.916), indicating that the collection analyzed is very diverse.

A few isolates were initially classified as non-typeable by serotyping because of positive agglutination with both types II and III sera when using sera from Denka Seiken. Serotyping of these isolates by the capillary precipitation method (using Statens Serum Institute sera) and by a latex agglutination assay using Essum Probiotics sera, revealed that in all cases the isolates only presented a positive reaction with serotype II specific serum. This phenotype was genetically confirmed by RFLP and Southern hybridization (the latter excluding the possibility of the strains carrying two capsular loci), further confirming that the correct identification was serotype II. With this in mind, all isolates previously classified as either serotype II or III using Denka Seiken sera were independently confirmed using either of the other sera and conventional serotyping methodologies to exclude any possibility of error. We believe these observations are the result of cross-reaction between type II and III sera, a possibility admitted in the latest literature provided by the manufacturer. This can lead to errors in serotyping if not further confirmed by other sera or typing methods.

In general, serotype was associated with the overall genotype defined by PFGE. The Wallace coefficient (W) relating the PFGE clusters with the serotype (W=0.720, $CI_{95\%}$ 0.666 to 0.775) was high, indicating that isolates grouped in the same PFGE cluster frequently also share the same serotype. However, some clusters were found to contain isolates with serotypes that were different from the most frequently found serotype in the cluster, suggesting that capsular switching could have occurred (n=27/463, 5.8%). Confirmation of the serotype of these isolates using conventional serotyping methods revealed in 18 cases that the original serotype was in error. However, we did confirm the serotype of nine isolates (n=9/463, 1.9%) that differed from the most prevalent serotype in the same PFGE cluster and these cases were further studied (Figure 6.1).



	No. of isolates (n=463)	Prevalent serotype	Capsular transformant (serotype)[a]
A	6	III	-
B	22	V	406301 (IV)
C	57	V	351726 (IV)
D	7	III	-
E	45	III	-
F	7	III	-
G	100	Ia	SH366 (II) 2227 (III) 312754 (Ib) 927045 (V)
H	5	Ia	-
I	22	Ib	CN4609 (Ia)
J	8	V	-
K	10	II	-
L	9	II	-
M_1	27	II	316560 (III)
M_2	38	III	-
N	22	II	-
O	5	V	-
P	6	III	931422 (V)

FIGURE 6.1 Dendrogram analysis of the PFGE profiles of 463 GBS isolates. UPGMA and the Dice coefficient (indicated as percentages in the scale above the dendrogram) were used to construct the dendrogram. Each PFGE cluster (defined as a group of ≥5 isolates with a Dice coefficient of ≥80% in the dendrogram) is represented by a triangle with size proportional to the number of isolates included in the cluster. The clusters are identified by capital letters outside each triangle. Putative capsular transformants were selected based on exhibiting a serotype different from the dominant serotype of the PFGE cluster. M_1 and M_2 are two sub-lineages of the same cluster, defined at >80% similarity in the dendrogram.

Every putative transformant was compared to two other isolates in the same PFGE cluster having the prevalent serotype of the cluster and PFGE profiles as similar as possible (maximum of three different bands). Each set of three isolates (the putative transformant and the two isolates used for comparison) was characterized for genes distributed across the GBS genome (Figure 6.2) and for confirmation of the genetic determinants of the capsular polysaccharide.

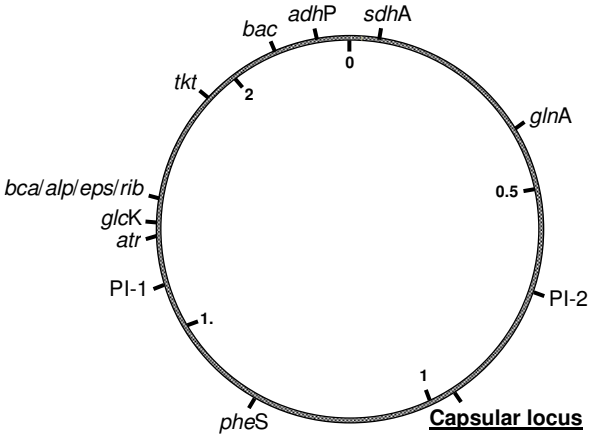


FIGURE 6.2
Genome map of *S. agalactiae* indicating loci of interest. Relative positions of housekeeping genes characterized in the MLST scheme (*phe*S, *atr*, *tkt*, *gln*A, *sdh*A, *glc*K and *adh*P), genes identified for surface protein gene profiling (same relative position for *bca, alp*2, *alp*3, *alp*4, *rib*, or *eps*), and *bac*, regions characterized for pili-island profiling PI-1 and PI-2 (same relative position for PI-2a or PI-2b), and the capsular locus region are depicted. Numbers inside the circle represent the scale in megabases.

MLST, surface protein gene profiling, and pilus-associated gene profiling

In the nine sets of isolates studied the STs found (Table 6.2) were associated with serotypes in agreement with previous observations (17, 21, 25). With no exceptions, all isolates were positive for only one alpha or alpha-like protein gene and occasionally the isolates were also positive for the β-antigen gene (Table 6.2). None of the isolates tested were positive for the *alp*2 or *alp*4 genes. The isolates were also tested for the presence of the genetic determinants of the pilus-like structures, and all were found to contain at least one of the identified islands. The PI-2a variant was present in

all isolates, whereas PI-1 was found together with PI-2a in 16 isolates and none were positive for the PI-2b variant (Table 6.2).

Sequencing of the conserved regions of the *cps* locus

All 55 single nucleotide polymorphisms and one repetitive sequence suitable for molecular serotyping in the *cps*D-*cps*G region described previously (18) were analyzed, and a molecular capsular type was assigned to each isolate according to this methodology. A similar analysis was done for the *neu*B-*neu*A region. The single nucleotide polymorphism patterns found were characteristic of the serotype identified using conventional immunological techniques for all isolates except strain 312754 in set S3 (Table 6.2).

Southern hybridization and capsule gene cluster RFLP analysis

The Southern blot hybridization methodology used to probe the capsular locus aimed at determining if a serotype-specific gene of the *cps* locus was present in each isolate and, if so, its location in the chromosome. This information was essential to exclude the possibility of "binary encapsulated" strains (1) or the generation of hybrid *cps* loci that could cross-react with two existing sera. We observed that all isolates tested presented only one capsular locus, as hybridization occurred with a single probe and in a single chromosomal fragment. We also observed that the positive hybridization fragment was of the same size in sets S1 to S5 and S7 to S8 but not for sets S6 and S9 (Table 6.2). In the latter cases one of the three different bands in the PFGE profiles of the isolates included the fragment with the capsular locus, suggesting a possible link between capsular transformation and the differences in PFGE profile.

TABLE 6.2 Detailed characterization of the putative transformants and other isolates from the same PFGE cluster.

Set	PFGE profile (cluster)	Isolate	Serotype	MLST (ST)	Surface protein gene profile	Sequencing[a]		RFLP *cps*G-*neu*A	Hybridization positive fragment (Kb)[b]	Hybridization Probe[b]	Pilus island gene profile
						*cps*D-*cps*G	*neu*B-*neu*A				
S1	Identical (G)	SH366	II	24	*bca*	II	II	II	450	II	PI-2a
		707618	Ia	24	*bca*	Ia	Ia	Ia	450	Ia	PI-2a
		12631	Ia	24	*bca*	Ia	Ia	Ia	450	Ia	PI-2a
S2	Identical (I)	CN4609	Ia	10	*bca* + *bac*	Ia	Ia	Ia	582	Ia	PI-1 + PI-2a
		275586	Ib	10	*bca* + *bac*	Ib	Ib	Ib	582	Ib	PI-1 + PI-2a
		321927	Ib	10	*bca* + *bac*	Ib	Ib	Ib	582	Ib	PI-1 + PI-2a
S3	Identical (G)	312754	Ib	24	*bca*	Ia	Ia	Ia[c]	436.5	Ia	PI-2a
		333425	Ia	24	*bca*	Ia	Ia	Ia	436.5	Ia	PI-2a
		401936	Ia	24	*bca*	Ia	Ia	Ia	436.5	Ia	PI-2a
S4	Identical (M_1)	316560	III	295	*rib* + *bac*	III	III	III	500	III	PI-1 + PI-2a
		768348	II	28	*rib* + *bac*	II	II	II	500	II	PI-1 + PI-2a
		850409	II	28	*rib* + *bac*	II	II	II	500	II	PI-1 + PI-2a
S5	One-band	406301	IV	2	*eps*	IV	IV	IV	300	IV	PI-1 + PI-2a
	difference	424176	V	1	*alp*3	V	V	V	300	V	PI-1 + PI-2a
	(B)	345352	V	1	*alp*3	V	V	V	300	V	PI-1 + PI-2a
S6	Three-band	351726	IV	2	*eps*	IV	IV	IV	300	IV	PI-1 + PI2a
	difference	314460	V	2	*eps*	V	V	V	339.5	V	PI-1 + PI2a
	(C)	371990	V	2	*eps*	V	V	V	339.5	V	PI-1 + PI2a
S7	Three-band	927045	V	23	*eps*	V	V	V	582	V	PI-2a
	difference	401207	Ia	23	*eps*	Ia	Ia	Ia	582	Ia	PI-2a
	(G)	314927	Ia	23	*eps*	Ia	Ia	Ia	582	Ia	PI-2a
S8	Three-band	931422	V	19	*eps* + *bac*	V	V	V	582	V	PI-1 + PI-2a
	difference	950541	III	19	*rib*	III	III	III	582	III	PI-1 + PI-2a
	(P)	880372	III	19	*rib*	III	III	III	582	III	PI-1 + PI-2a
S9	Three-band	2227	III	396	*rib*	III	III	III	535.5	III	PI-1 + PI-2a
	difference	SH436	Ia	24	*bca*	Ia	Ia	Ia	500	Ia	PI-2a
	(G)	342399	Ia	24	*bca*	Ia	Ia	Ia	500	Ia	PI-2a

[a] The capsular type compatible with the single nucleotide polymorphisms and insertions found using the methodology described in the text is indicated.
[b] That is, the size of the fragment in the PFGE profile that presented a positive hybridization signal and the corresponding serotype identified by the probe (see the text).
[c] One fragment different from the characteristic serotype Ia RFLP-profile.

RFLP of the *cps* region was concordant with the phenotypically determined serotype in all isolates with the exception of isolate 312754 (phenotypically serotype Ib), whose pattern was different from the predicted pattern for serotype Ib isolates. In fact, the *cps* RFLP pattern was very similar to the one expected for serotype Ia isolates, with the exception of a single fragment (1,135 bp), which was smaller in strain 312754. An *in silico* analysis suggested that this fragment corresponded to the *cps*G-*cps*H region. Sequencing of this region showed two deletions (one of 197 bp and a smaller one of 6 bp) in isolate 312754 that generated a smaller fragment (932 bp instead of 1,135 bp) in the restriction profile. The deletion resulted in the fusion of the *cps*G and *cps*H genes without compromising the reading frame, and leading to the possible production of a fusion protein lacking 23 amino acids of the C-terminal region of *cps*G, 9 amino acids of the N-terminal region of *cps*H, and 2 amino acids close to the C-terminal region of *cps*H (Figure 6.3).

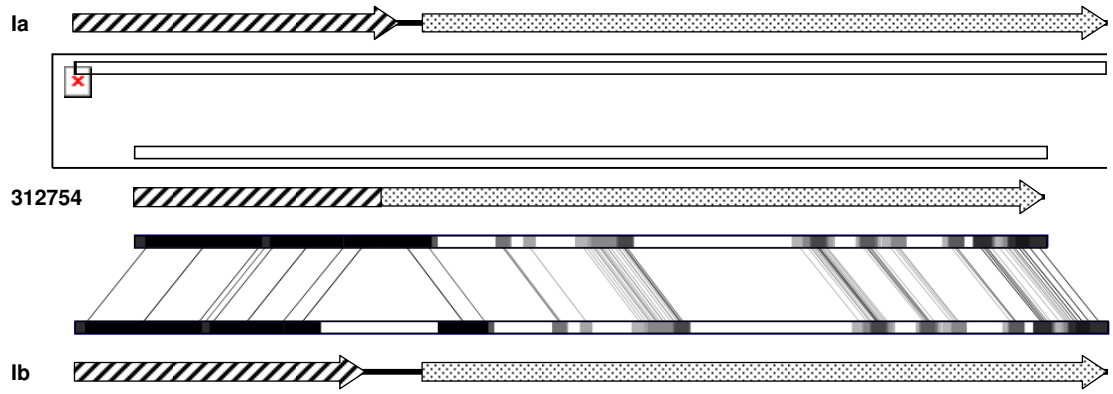


FIGURE 6.3 Diagram representing the DNA sequence of the *cps*G-*cps*H region of the capsular locus of *S. agalactiae* serotypes Ia and Ib compared to that of strain 312754. The *cps*G and *cps*H genes are represented by dashed and dotted arrows, respectively. The *cps*G-*cps*H fusion in strain 312754 is predicted to generate a fusion protein since the reading frame is maintained (see the text). The fragments with high DNA identity are represented by black boxes, whereas lower identity fragments are represented by lighter shades of gray. Similar fragments are connected by lines.

Discussion

Clonal structure of the *S. agalactiae* population

PFGE analysis showed a diverse population of GBS isolates with 16 different PFGE clusters identified (Figure 6.1). Such diversity is in agreement with the isolates being collected from multiple sources, in different years and age groups and associated with different severity of infections and colonization. Nevertheless, we observed that most isolates were grouped in clusters sharing the same serotype, implying an association between the serotype and the PFGE-based genotypes reflected in a high value of the Wallace coefficient and this coefficient would be even higher if we excluded non-typeable isolates (W=0.838, $CI_{95\%}$ 0.804 to 0.872). Non-typeable isolates frequently produce small amounts of capsule polysaccharide that easily revert to fully encapsulated variants (36) or result from mutations or from the insertion of mobile genetic elements in the *cps* locus, leading to absent or reduced expression of the capsule polysaccharide due to loss of function of the proteins involved in its biosynthesis (33). Thus non-typeable isolates probably do not represent capsular switching events but carry *cps* loci very similar to those of other isolates in the same PFGE cluster. Therefore, their inclusion in the calculation of the Wallace coefficients leads to an artificial underestimation of the concordance between the classification in PFGE clusters and serotype.

Still, in certain PFGE clusters, the presence of isolates whose serotype is different from the prevalent serotype in the cluster, but having similar PFGE profiles, suggested that capsular switching by horizontal gene transfer of the capsular genes could have recently occurred. The number of putative capsular transformant isolates in the present study was much larger than when we first analyzed the PFGE profiles (5.8%), and this value was in line with previous studies that addressed this issue (21). However, only nine isolates (2% of the total isolates) could be confirmed by a second round of traditional serotyping using different methods and sera. This significant decrease in putative capsule switching events can be explained by the fact that serotyping using phenotypic methods is particularly prone to errors due to user-dependent evaluation of an agglutination reaction that relies heavily on the experience of the user. On the other hand, the specificity of the polyclonal sera used may also be an issue in particular situations, as discussed previously. In fact, a validation study to evaluate the quality of serotyping of *S. pneumoniae*, another species of the

Streptococcus genus, in European reference laboratories identified a 5% error rate (20). Similarly, our data argue that serotyping errors in *S. agalactiae* may be frequent and contribute significantly to overestimate capsular switching events in this species. However, studies using capsular genotyping, and therefore not subjected to these errors, have also found evidence of capsular switching (32).

We used the classification in PFGE clusters as the main screening method to identify putative transformation events. To further identify recent capsular switching events, we characterized isolates with no more than three different bands in their PFGE profiles, which could result from a single genetic event meaning that the isolates are very closely related (42). For capsular switching to have occurred recently, the isolate with an unusual serotype must have been identical to other isolates of the same bacterial clone, all sharing the same genotype as defined by MLST, surface protein and pili island gene profile.

Capsular switching

Table 6.2 summarizes the characterization of the nine isolates identified as potentially resulting from capsular switching. Whereas some of the isolates had exactly the same genotype as the isolates they were being compared to (sets S1 and S2), other capsular transformants were found having different genotypic properties compared to additional isolates in the same PFGE cluster (sets S3 to S9). Sets S1 and S2 provide strong evidence for recent capsular switching since in these cases the potential transformants share the same genotype as defined by PFGE, sequence type, surface protein gene and pilus island gene profile as the comparator isolates, with the only difference residing in the capsular locus.

Initially, we considered the possibility that SH366 had acquired only the type II-specific genes *cps*G-*cps*K, as proposed in the literature (9, 22), and so the capsular locus of the putative transformant would be expected to be a hybrid between the recipient and the donor *cps* loci. For instance, strain SH366 was expected to exhibit features of both serotype II (recently acquired central genes *cps*G-*cps*K) and Ia (conserved regions of the operon *cps*A-*cps*F and *cps*L-*neu*A). If that were so, we should find the type Ia-specific polymorphisms when sequencing the conserved regions of SH366 capsular locus. This was not the case, and the entire capsular locus of SH366 was consistent with the type II capsule. These observations led us to conclude that SH366 had acquired the entire

capsular locus coding for the type II capsule and that the recombination breakpoints are located outside of the capsular locus. An *in silico* analysis of the published GBS genomes showed that the regions flanking the capsular locus are extremely conserved among *S. agalactiae* isolates (data not shown); therefore, homologous recombination in these regions is possible independently of the overall genetic background of the isolates. Moreover, recent data describes the region surrounding the capsular locus as showing evidence of a high density of recombination events corroborating our data, while identifying the transfer of DNA fragments as large as 334 Kb (5). The sequence type of SH366 (ST24) is almost exclusively found among serotype Ia isolates (16, 21), with a high prevalence in Portugal (25), and accounts for one of two main lineages of serotype Ia (unpublished data). A similar situation was found in set S2, with strain CN4609 possessing a *cps* locus entirely consistent with serotype Ia but found in a PFGE cluster of serotype Ib isolates, indicating the acquisition of a large chromosomal fragment encompassing the entire *cps* locus (Table 6.2). CN4609 is a representative of ST10, frequently found among serotype Ib isolates but previously not described in serotype Ia isolates (16, 21, 25), further supporting its identification as a capsular transformant. Taken together, these findings provide evidence of recent capsular switching events in SH366 and CN4609.

In set S3, we found that strain 312754 (phenotypically identified as serotype Ib) presented two deletions in a *cps* locus that was closely related to that of serotype Ia (Figure 6.3). These deletions result in the loss of 23 amino acids in the *cps*G gene product, a β-1,4-galactosyltransferase, and of 11 amino acids in the *cps*H gene product, the capsular polymerase, with the consequent production of a 505 amino acid fusion protein. Nuclear magnetic resonance studies showed that the types Ia and Ib polysaccharides have an identical composition and differ only in the glycosidic bond of the galactose residue to the N-acetylglucosamine in the lateral chain – type Ia polysaccharide has a β(1→4) bond and type Ib polysaccharide a β(1→3) – and these bonds are critical for the immune specificity of both polysaccharides (15). The characterization of the genes that compose the capsular locus of type Ia attributed to each gene a specific role in assembling the capsular polysaccharide (44). The two β-1,4-galactosyltransferases identified were encoded in the *cps*G and *cps*J genes, responsible for the glucose-galactose bond in the polysaccharide backbone and the galactose-N-acetylglucosamine bond of the lateral chain, respectively (44). Since this last bond is the

only difference between type Ia and Ib capsules, and considering that the *cps*J gene is not altered in 312754, it would be expectable that, in this isolate, the merging of *cps*G and *cps*H genes would result in the production of an Ia or Ia-like capsular polysaccharide. However, the data presented here seems to argue that *cps*G would be responsible for the β-(1→4)-galactose-N-acetylglucosamine bond. The *cps* locus of strain 312754 is therefore closely related to that of serotype Ia isolates (Figure 6.3) not being clear, in the light of previous knowledge, how the deletions described above justify the expression of a type Ib capsule. Discrepant results between phenotypic serotyping and the capsular locus were described previously for a GBS isolate (19), but no detailed study of its capsular locus was performed. It is also possible that the capsular polysaccharide expressed by strain 312754 is biochemically different from both type Ia and Ib capsules but is somehow recognizable by the Ib polyclonal serum used in conventional serotyping; however, a detailed characterization of this polysaccharide is outside the scope of the present study.

In sets S4-S9 the isolates express a serotype that is unique in the cluster and show different genetic features when compared to other isolates of the same PFGE cluster. These differences are shown in Table 6.2 and include MLST sequence type (sets S4, S5 and S9), PFGE profile (sets S5 to S9), surface protein gene profile (sets S5, S8 and S9) and the presence of the pilus islands-associated genes (set S9). All the markers used to evaluate the genetic background of the isolates are distributed in an arrangement conserved across serotypes and spread throughout the GBS genome (Figure 6.2), assuring that if horizontal gene transfer occurs these should be independently transferred among isolates providing a good measure of the genetic background against which to detect capsular transformation.

In set S4 the isolates have different sequence types (STs) and share the remaining features tested. ST295 and ST28 belong to the same clonal complex (CC19) and are double-locus variants, presenting different alleles for the genes *adh*P and *gln*A. Since the distance between these genes is more than 400 Kb (Figure 6.2) and each is even further distant from the capsular locus, it is unlikely that the event driving capsule switching and the acquisition of the two alleles occurred simultaneously. This implies that at least three independent genetic events occurred between the putative transformant and its ancestors.

In set S5 the putative capsular transformant and the representatives of the cluster show differences in the PFGE profile, the MLST profile, and surface protein gene profile. ST1 and ST2 are single-locus variants in the same clonal complex (CC19) and differ only in the *atr* allele. The *eps* and *alp*3 genes code for epsilon and alpha-like 3 proteins, respectively; both are members of the alpha-like surface protein family and mutually exclusive that have been mapped to the same genomic location. Considering that the surface protein and the *atr* gene are ~ 64 Kb apart, both of these genes could be transferred among isolates in the same DNA fragment. In this case, a minimum of two independent genetic events would have to be invoked to explain the differences observed, between the putative transformant and its ancestors.

Sets S6 and S7 include isolates that share all the characteristics tested with the exception of the PFGE profile, that differs in three bands between the putative capsular transformants and the representatives of the PFGE cluster where they were found. In set S6 some of the detected differences in the PFGE profile involve the fragment where the *cps* locus is found, as demonstrated by Southern hybridization, suggesting that the capsular switching itself could be linked to the changes in the PFGE profile. A single genetic event, involving recombination of a large DNA fragment, could result in both capsular switching and a different PFGE profile. This hypothesis is supported by the successful incorporation of DNA fragments up to 334 Kb in *S. agalactiae* (5). In set S7 the changes in the PFGE profile of the putative transformant do not involve the fragment containing the capsular locus, not suggesting a direct link between the changes in PFGE profile and *cps* locus, although this cannot also be formally excluded.

In set S8 the isolates differ in their PFGE profiles by three bands and in the surface protein gene profiles. Again since the genomic locations for the *eps* or *rib* genes and the *bac* gene are more than 350 Kb apart and even more distant from the capsular locus, all of these genes should be independently transferred among the isolates. Any of these genetic events could also give rise to the differences observed in the PFGE profiles, but a minimum of three independent recombination events would still be needed to explain the differences between the isolates.

Finally, set S9 comprises isolates showing very different genetic properties and thus unlikely to be closely related. These differences include the PFGE profile, all seven MLST alleles, surface protein, and the pilus island gene profiles. The grouping of these

isolates by PFGE seems to have been spurious, and all other evidence argues against capsular transformation.

Capsular switching is thought to contribute to the rise of new serotype-genotype combinations, allowing evasion of immune pressure. However, the success of these new variants is possibly restrained by interactions between the new capsule and the original properties of the isolate. If immune pressure were an overwhelming selective force, we would expect to see the expansion of these capsule switching sub-lineages. We did detect in our sample a PFGE cluster that contains two sub-lineages, identified as such by PFGE (i.e., defined as two independent groups of isolates related at >80% similarity in the dendrogram), presenting different serotypes, STs, and surface proteins, suggesting the existence of stable related lineages (Figures 6.1 and 6.3, PFGE cluster M). However, after confirmation of the initial serotyping results, we found only nine isolates that met our initial criteria and, upon more detailed analysis, only a fraction of these could result from recent capsular switching events. This could be due to either a small rate of capsular transformation, the clearing of these variants from the population or a combination of both. The stability of several GBS clones, such as the hypervirulent ST17 clone found among serotype III isolates that is characterized by a specific combination of genetic markers including mobile genetic elements and virulence genes, that are part of the variable genome and thus not shared by all GBS (2, 4, 21), suggests that the rate of gene exchange in this species may be lower than that of other streptococci. In fact, the PFGE analysis of collections of *S. agalactiae* isolates shows fewer clones than in other streptococcal species, such as *Streptococcus pyogenes* (14) and *S. pneumoniae* (37), consistent with a less diverse clonal structure of the population. On the other hand, genomic analysis of eight fully sequenced strains of *S. agalactiae,* provided evidence for the exchange of large chromosomal fragments, hinting at a high rate of gene exchange through an unusual mechanism (5).

The analysis presented here used several loci and took into account both the core and variable portions of the genome when analyzing GBS isolates. This approach is expected to allow us to identify changes in the genomic background of the transformants with high resolution, enabling the identification of recent capsular switching events. Our data suggest that serotyping errors may account for a significant proportion of putative capsular transformation events described previously, reinforcing the importance of different methods and sera or a genetic approach in confirming all

putative transformation events. Our analysis also provided unambiguous evidence for the existence of capsular transformation in GBS and indicated that the exchange of the entire capsular locus is more widespread than switching restricted to capsule-specific genes. Taken together, our data support the existence of capsular switching in GBS but at a lower frequency than previously suggested.

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CHAPTER 7

Concluding remarks

Group B streptococcus (GBS) is primarily a colonizing agent of the genitourinary and gastrointestinal tracts, yet it has long been recognized as an important agent of invasive disease in neonates. Implementation of screening guidelines and effective intrapartum antimicrobial prophylaxis (IAP) have contributed to significantly reduce neonatal infections in the early-onset period; however late-onset infections mostly remained unchanged. In Portugal, nearly all hospitals are now expected to follow the recommendations for prevention of perinatal GBS infections from the Portuguese Society of Paediatrics (2), even though no information has been issued from official health authorities about the implementation and effectiveness of these measures. Therefore, follow-up on GBS infections has been made mostly at the hospital level; aside from one study based on national voluntary reporting of neonatal infections (16). A first study addressing the prevalence of capsular types and antimicrobial susceptibility patterns in the largest Lisbon hospital (8), was available at the beginning of the studies included in this thesis, but in fact very little was known on the epidemiology of GBS infections in Portugal, and even less on the molecular properties of the isolates.

This lack of information substantiated the importance - and main purpose of this thesis - that of the epidemiological characterization of the GBS isolates causing neonatal infections in Portugal, as well as of those responsible for an increasing number of invasive infections in non-pregnant adults. The availability of new molecular typing techniques, alongside the classical phenotypic methods contributed to the improved characterization of GBS populations. The analysis of the data provided by these studies helped determining the main GBS lineages circulating in Portugal, and put them in the context of the worldwide GBS population causing infections in humans, hopefully bringing novel insights into the population dynamics of this pathogen.

GBS epidemiology in Portugal

Overall, the GBS isolates recovered from colonization in pregnant women as well as those causing invasive disease in Portugal were very diverse, some having the capacity of occupying multiple niches and found asymptomatically colonizing the vagina and rectum as well as causing mild to severe infections in neonates and adults. While the wide range of serotype-genotype combinations found in the studies presented in this thesis corroborates this diversity, it is apparent that simultaneously a clonal

structure can be found among the GBS isolates in Portugal, since specific lineages are not only more frequent in the population but are also more likely to cause invasive infections or to be associated with particular age groups. Still the GBS diversity was not constant in all age groups.

The multiplicity of genetic lineages observed in non-pregnant adults was higher than that found among invasive isolates from neonates, which presented with a substantially more clonal genetic structure.

Accordingly, GBS invasive infections in non-pregnant adults had a broader spectrum of disease presentation and were more frequent in the elderly, reflecting the possible impact of risk factors that increase with age, such as co-morbidities and immune senescence. In addition, men were found to have a higher susceptibility for GBS disease, regardless of age. Yet the reasons behind the sex imbalance remain to be clarified.

Serotype distribution

As seen in most countries, even though nearly all serotypes are able to both colonize and cause invasive disease, the prevalence of the GBS serotypes was different according to disease presentation and age groups (Figure 7.1).

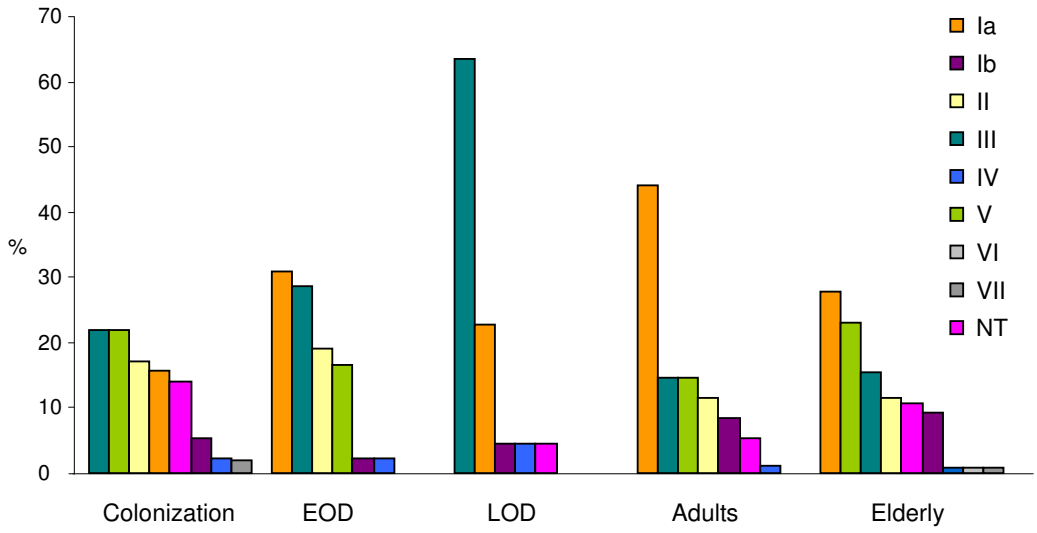

FIGURE 7.1 Relative prevalence of GBS serotypes among colonizing and invasive isolates in different age groups in Portugal. Colonization (pregnant women), EOD (0-6 days), LOD (7-90 days), Adults (18-64 years), Elderly (>64 years). Adapted from Chapters 2 and 5.

The comparison of the prevalence of serotypes in different human populations revealed some interesting findings. First, maternal colonization includes a wide range of serotypes, with capsular types III and V being more frequent but immediately followed by serotypes II and Ia. Considering that vertical transmission accounts for the majority of neonatal disease cases, particularly during the early-onset period, our data is in agreement with a maternal source of infection, with the leading serotypes colonizing pregnant women also being responsible for most EOD cases. Also worth mentioning is importance of serotype Ia among neonatal infections, that in Portugal was found to be associated to EOD. Among the GBS isolates recovered from LOD, serotype III was overwhelmingly predominant, similarly to what has been described elsewhere (15). It accounted for nearly two thirds of the isolates recovered in the late-onset period, and was frequently found in the cases of meningitis.

While maternal GBS carriage is a prerequisite for EOD, the source for disease among adults is poorly understood, possibly reflecting asymptomatic colonization (20). Among the adult populations the distribution of the GBS serotypes was again different and more varied than that from neonatal invasive disease, which is consistent with a more diverse spectrum of disease presentations and frequent underlying medical conditions found in these patients. Nevertheless, serotype Ia was dominant, particularly among younger adults (18-64 years old), and these results reflect a geographical specificity, in which Portugal is clearly different from other European countries and the United States, where serotype V prevails among invasive infections in younger and elderly adults (13, 21). The reasons behind the higher prevalence of serotype Ia in these age groups in Portugal remain unclear, but the genetic lineages responsible for these infections are the same that cause invasive disease in neonates.

Finally, other relevant information that can be drawn from Figure 7.1 is the number of non-typeable (NT) isolates, particularly high in the colonizing isolates and increasing with age among the adults. The polysaccharide capsule of GBS is an important virulence factor that contributes to the bacterial evasion of the innate immune system of the host. Consequently, the majority of the strains causing invasive infections, particularly in newborns, are encapsulated. The data in Figure 7.1 are consistent with previously reported higher prevalence of NT isolates among colonized women compared to neonates (7), and further supported by the low invasive disease potential described for the NT isolates among neonates in Portugal. The prevalence of NT

isolates in pregnant women as compared to the neonates suggests that these isolates are maybe less fit to cause disease, and the possibility exists that these isolates remain as colonizers throughout the adult life, thereby explaining their higher prevalence in infections in adults. Furthermore, it has been speculated that capsule expression may be regulated in response to host environment, and that non capsulated isolates can more easily adhere to epithelial cells, because the capsule hinders the interaction of bacterial surface factors with the epithelial cell receptors (17).

In adults, particularly the elderly, it is possible that a colonization microenvironment particularly selects for less capsule production, thus favoring persistence of non-typeable GBS (18), yet it is unclear if colonization is the reservoir for GBS infections in adults. The diversity of underlying conditions that result from the natural ageing process, together with the immune senescence of the hosts, probably provide GBS with more opportunities to cause invasive disease regardless of the bacterial genetic background, including the lack of a polysaccharide capsule that is believed to be a major virulence factor.

GBS genetic lineages

While many studies concerning the clonal composition of the GBS population have identified the more significant serotypes and genetic lineages associated to invasive disease, still differences have been found in different countries reflecting a perhaps slower but dynamic nature of this pathogen, and further supporting continuous surveillance.

Some major genetic lineages have been detailed in recent years, as result of the combinations of phenotypic methods with multiple genotyping systems. In the studies described in this thesis, the main lineages associated with invasive infections in newborns and adults were described, and found mostly to be in agreement with those circulating in other countries. However, additional data presented here highlight the importance of local dynamics, indicating that genetic evolution of GBS presents with a geographic structure and may depend on local factors.

Among neonates, two main genetic lineages were responsible for invasive infections. In EOD cases, serotype Ia isolates were more prevalent, grouping together in a major PFGE cluster constituted by two genetic sub-lineages represented by ST23 and ST24. While ST23 is recognized as the main genetic lineage of serotype Ia, ST24 has

been rarely found elsewhere and an unusually high proportion of this lineage was first reported in the study. Among the LOD cases, serotype III was prominent. Since the availability of an MLST scheme for GBS, the serotype III genetic lineage ST17 has been described worldwide under the designation of hypervirulent clone, and associated to an increased capacity of causing neonatal invasive disease (12). The GBS isolates recovered in Portugal were no exception, mostly belonging to this genetic lineage, but were significantly associated with both EOD and LOD.

Chapter 3 includes the analysis of a large collection of GBS isolates recovered from neonatal invasive infections in Barcelona, Spain. It represented an interesting study in the context of this thesis due to the opportunity to perform, under the same molecular typing methodology, the characterization of the a large number of invasive GBS isolates circulating in another geographic location, that still had enough proximity to eventually present analogous results.

Indeed, the clonal structure and diversity of genetic lineages found in Barcelona was similar to those of Portugal, validating the comparative analysis of both studies. In the investigation of the GBS isolates from Barcelona, the same two major genetic lineages were identified among neonatal isolates; again an association between III/ST17 and LOD and a significant proportion of serotype Ia isolates represented by ST23 and ST24. The main discrepancies concerned the associations between the genetic lineages and disease presentation, in Barcelona Ia/ST23/ST24 was not overrepresented among EOD and III/ST17 was only associated to LOD and not both types of disease onset. These differences are probably due to the methodological differences between the two studies (no colonizing isolates were available from Barcelona) and eventually to the smaller number of GBS isolates recovered in Portugal.

Furthermore, in the serotype Ia sub-lineages, a complete association was found between the MLST-based sequence types and surface protein genes, with all ST23 isolates carrying the *eps* gene and all ST24 isolates exclusively the *bca* gene, further discriminating both lineages. While supporting for the circulation of both sub-lineages in Portugal, these results also suggest that the presence of a particular Alp family surface protein gene is rather a clonal property than a feature of the serotype.

The case report analysis of two clinical cases of meningitis in adults in Portugal (Chapter 4) was not part of the initial work plan composing this thesis, but rather posed

as an interesting opportunity to identify a possible community outbreak of GBS disease. The detailed investigation of the isolates responsible for these meningitis cases revealed a similar genetic background for the isolates as defined by their identical serotype Ib, PFGE profiles, ST10 and later determined surface protein gene *bca*. These data supported a common source for the GBS isolates, emphasized by the temporal and geographical clustering of the cases. However, despite the efforts from the Portuguese public health authorities, also involved in this investigation to identify the source of the isolates, no such result was achieved. The virulence of the bacterial strain causing these infections, suggested by the death of one of the patients and significant morbidity in the other case, in spite of appropriate diagnosis and antimicrobial therapy, reinforce the importance of surveillance while raising questions as to the possibility of community outbreaks of GBS infections of unknown sources.

Chapter 5 comprised the study of the GBS isolates causing invasive disease among adult and elderly populations in Portugal, in which a more diverse array of genetic lineages were found. As opposed to the United States and most European countries where serotype V prevails among invasive infections in non-pregnant adults, in Portugal it accounted for less than 20% of the GBS isolates, and was nearly as frequent as serotype III. While no particular lineages of serotype III were evidenced, serotype V was mostly associated to two genetic lineages represented by ST1/*alp*3 and the infrequent ST2/*eps*. Additionally, the distinction of the serotype II genetic lineage ST28/*rib* was also proposed in this study, and suggested to have a possible tropism for joint infections, considering its overrepresentation among the isolates recovered from synovial fluid.

Once more, a major difference between the Portuguese and other studies was the PFGE cluster including most serotype Ia isolates, and represented by ST23/*eps* and ST24/*bca*, further confirming the high prevalence of the later in Portugal. Nevertheless, among the serotype Ia isolates, a new lineage was found in a different PFGE cluster, that grouped together a set of isolates characterized by Ia/ST8/*bca* and serotype Ib/ST10 and related STs all presenting the surface protein gene *bca*. Interestingly, ST8 had already been identified, but in that instance among serotype Ib isolates, in the analysis of the two cases of meningitis in adults (Chapter 4). Considering that ST8 and ST23/ST24 are unrelated STs, and that this lineage was not found in previous studies, in

which serotype Ia isolates presented a remarkable clonal structure in terms of the genetic lineages defined by both PFGE and MLST, these data suggest that this lineage is the result of relatively recent capsular switching events. The new serotype-genotype combination has probably an adaptive advantage at causing invasive infections in adults, and this could explain why the Ia/ST8/*bca* lineage was not associated with vaginal colonization or neonatal infections.

Considering the results from the characterization of the GBS isolates in all age groups, a major finding of these studies is the geographical expansion of the Ia/ST24/*bca* sub-lineage in Portugal, further strengthened by the investigation performed with the Barcelona isolates, and another report from Italy (10) that suggest a Mediterranean dissemination.

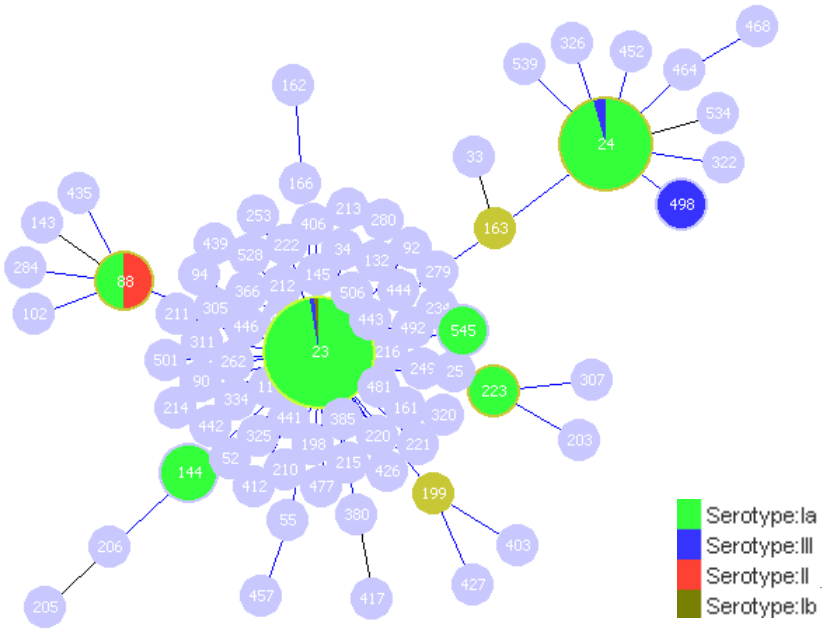

FIGURE 7.2 Representative diagram of goeBURST group 2, including all isolates recovered from colonization and invasive disease in neonates (Chapters 2 and 3) and adults (Chapter 5). Light blue circles indicate STs that are not present in the collection analyzed, light brown STs are sub-founders identified has having more than three connections in the goeBURST diagram. The size of the circles (except the light blue and light brown) is proportional to the number of isolates (in a logarithmic scale). Figure prepared using the PHYLOVIZ software (http://goeBURST.phyloviz.net).

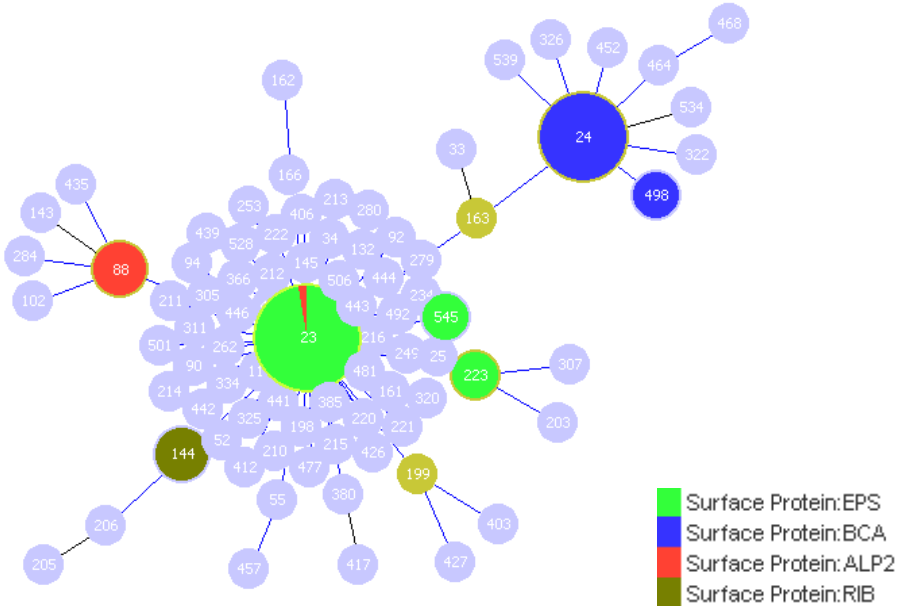

FIGURE 7.2 (continued)

The nearly exclusive association of ST23 and *eps*, and of ST24 and *bca*, and the very small number of isolates with other capsular types presenting these and related STs (Figure 7.2), argue for a contemporary clonal expansion of a specific sub-lineage within some geographical boundaries.

While the diversification of GBS populations is ongoing, continued monitoring of the genetic lineages responsible for GBS infections in the near future may yield important information for the comprehension of the population dynamics of this pathogen.

Antimicrobial resistance

Serotype V has long been recognized as the most frequent serotype causing invasive infections in non-pregnant adults in the United States and most European countries, to which the genetic lineage ST1/*alp*3 has added importance due to its association to macrolide resistance (19). In Portugal, erythromycin resistance was also more frequently among serotype V isolates, still only 40% of erythromycin resistance was found in this serotype, while the remaining was dispersed in most other serotypes. In fact, erythromycin resistance presented as a clonal property of V/ST1/*alp*3 instead of a specific feature of serotype V. The identification of another lineage of serotype V, ST2/*eps*, fully susceptible to several antimicrobials, including tetracycline, to which

GBS is nearly ubiquitously resistant, may have contributed for this clonal asymmetry of macrolide resistance in serotype V. In Barcelona, erythromycin resistance was associated to serotype V (30% of resistant isolates) but also distributed in various serotypes. The fact that among the neonates only the lineage V/ST1/*alp*3 was found, but not the susceptible V/ST2/*eps,* may indicate a strong host age-dependence of this lineage's ability to cause invasive disease.

Another particular finding in the study from non-pregnant adults in Portugal was the high number of isolates carrying the *erm*(TR) macrolide resistance gene, as opposed to a previous report from Portugal (8), and to the study of neonatal infections from Barcelona, in which macrolide resistance was more frequently determined by the *erm*(B) gene. Also, most reports from other countries are generally in agreement as to the dominance of *erm*(B) gene among erythromycin resistant isolates (4, 9, 11), so again local factors can be contributing to the spread of particular resistance determinants. Moreover, although all neonatal isolates presented the $cMLS_B$ phenotype, among adults erythromycin resistance was similarly distributed among the $cMLS_B$ and $iMLS_B$ phenotypes, while in previous studies from Portugal and other countries the $cMLS_B$ phenotype was much more prevalent than the $iMLS_B$ (4, 8, 9).

An origin for the spread of this resistance gene cannot be determined by a comparison of this and the previous Portuguese study (8), comprising invasive, non-invasive and colonizing isolates. If on one hand, the spread of the *erm* genes throughout most serotypes and clusters suggests a multiclonal dissemination of resistance, on the other hand the association between erythromycin resistance and particular genetic lineages (such as the V/ST1/*alp*3) supports a clonal expansion model of resistance. Nevertheless, considering the general consensus on the predominance of this genetic lineage in macrolide resistant isolates, yet carrying either the *erm*(B) or *erm*(TR) genes, it is possible that they constitute two sub-lineages that are not discriminated by the typing methods currently used to characterize these isolates. It is possible that the horizontal gene transfer of the *erm*(TR) gene into a genetic lineage previously carrying the *erm*(B) gene could have been advantageous for the isolates causing invasive disease among adults in Portugal, leading to their expansion and explaining the variations observed within the same geographic region.

Macrolide resistance rates in Portugal are not showing significant increasing trends, despite a supposed increment in macrolide use following the introduction of IAP

in recent years. Continuous surveillance of macrolide resistance will help clarify the mechanisms by which the dissemination of resistance occurs in GBS, and to which extent it depends on local genetic lineages.

The genetic determinants responsible for tetracycline resistance were assessed among the GBS recovered from non-pregnant adults in Portugal and, as frequently reported elsewhere, tetracycline resistance was mostly mediated by the *tet*(M) gene. This gene was present in all serotypes and major PFGE and MLST-based genetic lineages, in agreement with a multiclonal dissemination of resistance. It was also found together with the erythromycin resistance *erm*(B), *erm*(TR) and *mef*(E) genes, however not all erythromycin resistant isolates were simultaneously resistant to tetracycline, not being apparent the association between particular *erm* and *tet* genes, already shown to be carried by the same transposons in GBS (19). Interestingly, the few isolates carrying the *tet*(O) gene, frequently associated to bovine strains (6), were mostly clustered in a single PFGE cluster including different serotypes, proposing a clonal spread for this genetic determinant.

Capsular switching

Typing methods are now widely used to discriminate large collections of isolates based on their phenotypic and genotypic characteristics, in order to determine the genetic relationships between strains. However, the congruence between the different typing methods has proved challenging in face of the diversity of phenotype-genotype combinations found among GBS isolates. Comparative genomics of GBS introduced the concept of a “pan-genome” including a regulatory core genome, plus a variable genome consisting mostly of strain-specific genes, with abundant mobile and foreign elements, supporting the hypothesis that the majority of GBS specific traits depend on intra- and inter-species lateral gene transfer. Furthermore, the serotype-independent clustering of strains provided crucial evidence for capsular switching within this species as strains could share the same capsular type regardless of their genetic background (22).

The genetic heterogeneity found among GBS strains, even of the same serotype, proposed that the evolution of GBS is ongoing, particularly within the genes encoding virulence factors, including the capsular polysaccharide, due to recombination events leading to gene replacement or allelic exchange (3).

In the epidemiological studies, the absence of a complete correlation between serotypes and genetic the lineages defined by PFGE and MLST supported the hypothesis above mentioned, suggesting that capsular switching events could provide the explanation for the discrepancies found (5, 14). The study describe in chapter 6 benefits from previous investigations of large collections of GBS isolates performed in Portugal, in which similar inconsistencies in the relation of serotypes and genotypes were found. This study provided unequivocal evidence for the existence of capsular switching in GBS. These are, however, rare events; and their high frequency postulated previously was probably due to an overestimation resulting from serotyping errors. The proposed mechanism for capsular switching was by horizontal gene transfer of the whole capsular locus instead of the earlier suggested genetic transfer of only the serotype-specific genes.

Vaccines

Development of a universal vaccine is the most promising approach to the prevention of GBS infections, given the potential adverse effects of IAP, as well as the need for effective prevention of LOD and invasive disease in non-pregnant adults.

In the Portugal, a CPS-based vaccine against serotypes Ia, Ib, II, III, and V could potentially offer protection to 80-95% of the population including neonates, pregnant women and non-pregnant adults; however, the problem remains of pathogenic serotypes that are more prevalent in other parts of the world. Furthermore, NT isolates account for significant numbers among maternal colonization and invasive isolates in non-pregnant adults, and would not be covered by a polysaccharide-based vaccine formulation. The introduction of such a vaccine could also lead to serotype replacement as observed in *S. pneumoniae* (1), with the expansion of infrequent serotypes not included in the vaccine following a decrease in the infections caused by vaccine serotypes.

Finally, it is possible that vaccination will provide selective pressure for virulent genotypes to switch capsules and escape vaccine induced immunity. The work presented in this thesis identified capsular switching events in GBS, yet proposing for their rare occurrence. The selective pressure imposed by a vaccine could amplify these events in natural populations of GBS.

GBS surface structures other than the capsule hold promise as vaccine components by overcoming serotype-specificity and efficiently preventing infections. In

recent years, both capsule-protein conjugated and pilus-based vaccines have been developed, raising the possibility of prevention of both adult and perinatal disease. However, the PI-2a variant has been investigated in detriment of neonatally-associated PI-2b, and conjugate multivalent vaccines remain vague, therefore, a universal vaccine that could prevent all GBS disease is still elusive.

Considering that colonization is a pre-requisite for disease, and that GBS usually resides as a commensal microorganism, asymptomatically colonizing the human host in either a transient, intermittent or persistent way, it is crucial to extend our knowledge on the natural reservoirs of GBS, particularly those of the isolates causing invasive disease in the neonatal late-onset period and among adults, for the appropriate management of the GBS infections.

The studies presented in this thesis provided new insights into the population structure of GBS, emphasizing the diversity of the genetic background among different clinical conditions and age groups. Evidence was provided for the important role of recombination as a mechanism of genetic diversification in GBS. These findings, while contributing to a better knowledge of the global epidemiology of GBS, highlighted the importance of ongoing selection and local expansion of specific GBS clones, with significant impact on the dissemination of antimicrobial resistance or the spread of invasive clones into the community.

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