Molecular Epidemiology of Skin and Soft Tissue Infections by *Streptococcus agalactiae* in Portugal (2005-2016)

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Abstract

Group B *Streptococcus* (GBS) isolates causing skin and soft tissue infection (SSTI) in non-pregnant adults in Portugal were recovered from 2005 to 2016. The GBS isolates (n = 446) were characterized by capsular serotyping, surface protein and pilus island gene profiling. Antibiotic susceptibility testing was performed, and resistance genes were identified by PCR. The isolates were assigned to sequence types (ST) and clonal complexes (CCs) by multilocus sequence typing (MLST). Serotype Ia was the most frequent (31.8%), followed by serotype V (25.8%) and serotype Ib (15.7%). Throughout the study period a significant increase of serotype Ib was observed (p (CA) = 0.018), accompanied by a decrease of serotype Ia (p (CA) = 0.032). In 2016, serotype Ib became the most frequent serotype, being responsible for 25% of SSTI. Of all serotype Ib isolates, 59% clustered within CC1 (n = 41/70), which was the dominant CC, comprising most serotype Ib and 73% of serotype V isolates (n = 84/115). These isolates belonged to the CC1/alp3/PI-1+PI-2a genetic lineage, almost exclusively associated with serotype V. This new serotype/genotype combination resulted from a capsular switching event. In this recent genetic lineage there was an overrepresentation of the $cMLS_B$ phenotype (p (CA) < 0.001), present in 95% of the serotype Ib/CC1 isolates (n = 39/41). High-level streptomycin resistance was found in 6 isolates, that also presented the $cMLS_B$ phenotype. The emergence of a new genetic lineage highly resistant to macrolides and lincosamides, as well as the presence of multidrug resistant isolates, causing SSTI in non-pregnant adults in Portugal is troublesome. This data highlights the importance of GBS epidemiology in the monitoring of antimicrobial resistance of GBS infections.

Keywords: Group B *Streptococcus*; Skin and soft tissue infections; Non-pregnant adults; Molecular epidemiology; Genetic lineage; Antimicrobial resistance; High-level streptomycin resistance.

Introduction

Streptococcus agalactiae, also known as Group B *Streptococcus* (GBS), is a Gram-positive coccus and a known colonizer of the genitourinary and gastrointestinal tracts. GBS has been recognized since the 1960s as a leading cause of invasive infection in neonates, and a few decades later was also recognized for causing infections in adults (Schuchat, 1998; Schuchat and Balter, 2006). GBS infections are increasing worldwide, particularly in the elderly population (Alhhazmi et al., 2016; Lamagni et al., 2013; Morozumi et al., 2016; Skoff et al., 2009). This increase led to the need for vaccine development as a way to prevent GBS disease (Kobayashi et al., 2016). The capsular polysaccharide (CPS) is a major virulence factor and, so far, 10 capsular serotypes have been identified: Ia, Ib and II-IX. The identification of the CPS is frequently used to classify GBS isolates and essential for epidemiological characterization. The serotype prevalence is known to vary geographically and temporally. Together with the serotype, molecular methods, such as multilocus sequence typing (MLST), are used to discriminate GBS isolates into genetic lineages.

Certain genetic lineages and serotypes are responsible for most GBS infections. Hypervirulent clonal complex (CC) 17, usually associated with serotype III, was identified as the main contributor for invasive GBS disease in neonates (Manning, 2014).

In adults in the USA (Le Doare and Heath, 2013; Phares et al., 2008; Skoff et al., 2009), China (Wang et al., 2014) and Europe, serotype V has been predominant, usually associated with CC1 and macrolide resistance, although recently, other

serotypes have gained significance such as serotype III in Denmark (Lambertsen et al., 2010), Norway (Bergseng et al., 2008), France (Tazi et al., 2011), and Canada (Teatero et al., 2014). In Portugal, the dominant serotype contrasts with those found in other countries, where serotype Ia has been significantly more prevalent (Martins et al., 2012), and in England and Wales (Lamagni et al., 2013) serotype Ia is becoming more relevant. However, a change in serotype distribution was recently observed in Portugal, with the dominant serotype Ia decreasing and serotype Ib increasing (Lopes et al., 2018). In Japan, the most common capsular serotype was Ib (Morozumi et al., 2016).

In the case of GBS disease, penicillin is the antibiotic of choice for prophylaxis and treatment, although in case of penicillin allergy clindamycin is a suitable alternative. Cases of reduced penicillin susceptibility have been reported but the clinical impact of these findings is yet to be stated, nevertheless, the possible emergence of penicillin resistance in GBS is concerning (Kimura et al., 2008). Furthermore, macrolide and lincosamide resistance rates increasing have been reported worldwide (Alhhazmi et al., 2016; Castor et al., 2008; Lamagni et al., 2013).

The diversity of serotypes observed within the known genetic lineages and the emergence of new genetic lineages with certain characteristics highlights the need for continued surveillance.

The purpose of this study was to preform molecular epidemiological analysis on GBS isolates recovered from skin and soft tissue infections (SSTI) from non-pregnant adults, to identify the major genetic lineages and antimicrobial susceptibility related to GBS SSTI in Portugal.

Aim of the Study

In Portugal, the GBS population responsible for invasive disease in adults is well-known and well-characterized due to studies performed in the past years (Lopes et al., 2018; Martins et al., 2012), but, although SSTI is the most common disease presentation, no research has yet been done in Portugal regarding this subject. This study focuses on the GBS population responsible for SSTI in adults, with the main aim of complementing the research previously done in Portugal regarding invasive GBS disease in adults, providing an additional perspective of the lineages causing SSTI. This study will lead to an overall better characterization of the GBS population in Portugal, leading to the proper management and treatment of GBS infections, therefore highlighting the importance of epidemiological surveillance and epidemiologic studies.

In order to achieve this aim, the molecular characterization of GBS SSTI isolates was performed using phenotypic and genotypic methods. The phenotypic methods consisted of serotyping and AST, and the genotypic methods consisted of PCR gene profiling of surface proteins, pili and antimicrobial resistance, and MLST.

Materials and Methods

Bacterial Isolates

The GBS isolates were recovered from patients from 32 Hospitals and Hospital Centers throughout Portugal, as part of a laboratory-based surveillance program in which the hospitals' microbiology laboratories were asked to submit to a central laboratory all GBS isolates. For this study, an initial collection was assembled including all GBS isolates recovered from non-pregnant adults (≥ 18 years old) that presented skin and soft tissue infections (SSTI), over the period of 2005 to 2016 (n = 1774). From the initial collection, 25% of the isolates received each year were selected randomly, making up the study collection with a total of 446 GBS SSTI isolates.

Identification

The GBS SSTI isolates were identified to the species level in the microbiology laboratory of each hospital by standard methods. Confirmation of identification was done by latex agglutination using the Streptococcal grouping kit (Oxoid, Hampshire, England), according to the manufacturer's instructions.

Capsular Serotyping

Capsular serotyping of all GBS SSTI isolates was performed with the ImmuLexTM Strep-B kit (Statens Serum Institut, Copenhagen, Denmark), a serotyping kit based on a rapid latex agglutination test. The kit was used as indicated in the manufacturer's instructions.

Antimicrobial Susceptibility Testing

Susceptibility testing was performed using the Kirby-Bauer disk diffusion method and the procedures and interpretation criteria for Streptococcus spp. β-Hemolytic Group according to the CLSI 2015 guidelines (CLSI, 2015). The antibiotics tested included penicillin G, erythromycin, clindamycin, tetracycline, levofloxacin, vancomycin, and chloramphenicol. For the detection of HLAR the Kirby-Bauer disk diffusion method was also performed with streptomycin and gentamycin disks, according to the CLSI procedures and interpretive criteria for Enterococcus species (CLSI, 2015). Furthermore, the D-zone test was performed to determine macrolide and lincosamide resistance phenotypes: the MLS_B phenotype, corresponding to resistance to macrolides, lincosamides and streptogramin B, either inducible ($iMLS_B$) or constitutive ($cMLS_B$); the M phenotype, corresponding to resistance to macrolides only; and the LS_A phenotype, corresponding to resistance to lincosamides and streptogramins A.

DNA Extraction

Total bacterial DNA was extracted from GBS cells by treatment with mutanolysin and boiling.

Surface Protein Genes and Pilus Islands

For the surface protein genes, a multiplex PCR assay was performed to detect the *bca, eps, rib, alp2/alp3* and *alp4* protein genes, as described elsewhere (Creti et al., 2004). The *alp2* and *alp3* genes were differentiated as previously described (Martins et al., 2010). The presence of PI-1, PI-2a, and PI-2b was detected by PCR assay as described previously (Martins et al., 2010). The absence of PI-1 genes was confirmed by PCR as described elsewhere (Martins et al., 2010).

Resistance Genotypes

A multiplex PCR assay was performed on all macrolide resistant GBS isolates to detect the presence of the *erm*(B), *erm*(TR), and *mef*(E) genes, as described elsewhere (Figueira-Coelho et al., 2004), and an additional PCR assay was performed to detect the presence of the *erm*(T) gene (Compain et al., 2014). For lincosamide resistant GBS isolates, PCR assays were performed to detect the *lsa*(C) (Malbruny et al., 2011) and *lnu*(B) (Bozdogan et al., 1999) genes. Tetracycline resistant isolates were screened for the presence of the *tet*(K), *tet*(L), *tet*(M), and *tet*(O) genes, as previously described (Trzcinski et al., 2000). The presence of high-level aminoglycoside resistance (HLAR) genes, namely *aac(6')-aph(2"), aph(2")-Ib, aph(2")-Ic, aph(2")-Id, aph(3')-III, ant(4')-Ia* and *ant(6)-Ia* was performed by PCR (Clark et al., 1999; Vakulenko et al., 2003).

Multilocus Sequence Typing

MLST was performed as described previously (Jones et al., 2003) and sequence type (ST) assignment was done by using the *S. agalactiae* MLST database (http://pubmlst.org/sagalactiae). Analysis of DNA sequences was performed using the Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium). The goeBURST algorithm implemented in PHYLOViZ software (Nascimento et al., 2017) was used to establish relationships between STs. CCs were defined at the single-locus variant (SLV) or double-locus variant (DLV) levels.

Statistical Analysis

Simpson's index of diversity (SID) and 95% confidence intervals ($CI_{95\%}$) was used to estimate the diversity of the collection (www.comparingpartitions.info) (Carrico et al., 2006). The Cochran-Armitage test was used for trends and Fisher's exact test with false discovery rate (FDR) correction for multiple testing was used to evaluate differences (Benjamini and Hochberg, 1995). A $p < 0.05$ was considered significant for all tests. Information regarding the resident population in Portugal during the study period (2005-2016) was obtained from PORDATA and Instituto Nacional de Estatística (INE) (Resident population in Portugal (2005–2016): http://www.ine.pt and https://www.pordata.pt/ – accessed in July 2018.).

Results

Isolates

The GBS isolates were recovered in 32 Hospitals and Hospital Centers throughout Portugal from non-pregnant adults (≥ 18 years old) presenting SSTI, in the period of 2005 to 2016, making up a total collection of 1774 isolates. GBS were isolated from abscess, lesion or wound exudate (n = 1630), biopsy/tissue (n = 112) and ulcer (n = 32). In the total collection, 60% (n = 1064) of the isolates were recovered from male patients and 40% (n = 710) from female patients. The age range was 18–100 years old, averaging on 60 years old. In this collection, 58% (n = 1020) of the isolates were collected from young adults (18-64 years old) and 42% (n = 754) from elderly adults (≥ 65 years old). The overall number of infections per year increased (p (CA) < 0.001), not only on the elderly (p

(CA) < 0.001) but also on younger adults (p (CA) < 0.001), although there was a higher frequency of SSTI among elderly than young adults (overall incidence rate ratio (IRR) = 2.60, IC_{95}% 2.36-2.85). An overrepresentation of SSTI in young male adults was also found (p < 0.001). From the initial collection, a study collection was assembled for phenotypic and genotypic characterization, consisting of 25% of all GBS SSTI isolates recovered each year, selected randomly, making up a total of 446 bacterial isolates.

Capsular Serotypes

The study collection presented significant serotype diversity (SID = 0.789, CI_{95}% 0.771-0.807). Serotype Ia was the most frequent (31.8 %), followed by serotypes V (25.8 %), Ib (15.7 %) and III (12.3 %), together accounting for over 85% of the SSTI isolates. Of the 446 GBS isolates, 3.6% were non-typeable and serotypes VII and VIII were not detected. No statistically significant associations were found between serotype and gender or age group. Serotype IX was only found amongst the elderly, but this association did not reach statistical significance.

Regarding the serotype distribution, significant changes were observed throughout the study period (Figure 1). From 2005 to 2016 there was a substantial increase of the serotype Ib isolates (p (CA) = 0.018) and a decrease of serotype Ia isolates (p (CA) = 0.032). While serotype Ia was the dominant serotype in the first years of the study, serotype Ib became the most frequent serotype in 2016. Serotype V showed a statistically significant increase from 2005 to 2010 (p (CA) = 0.027) followed by a decrease from 2010 to 2016 (p (CA) = 0.014).

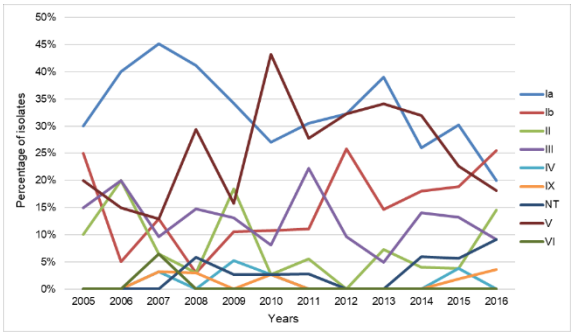

Figure 1 – Serotype distribution of the SSTI GBS isolates from 2005 to 2016.

Genetic Lineages - Multilocus Sequence Typing

According to MLST, the 446 SSTI isolates presented high genetic diversity, being distributed across 54 STs (SID = 0.898, CI_{95}% 0.882-0.915), with six being newly identified in this study (ST1199-ST1205). For one isolate the ST was not determined because the atr gene had a deletion of 408bp (between positions 19 and 426), to which an allele number was not assigned. The allele numbers of the other genes were assigned and were all similar to ST8, so this isolate clustered within CC12. The STs clustered into 9 CCs and two singletons, with lower genetic diversity, as expected (SID = 0.770, CI95% 0.749-0.790).

The predominant CCs were CC1 (33%, n = 146), CC23 (29%, n = 131) and CC19 (15%, n = 67). CC1 comprised most isolates of serotypes Ib (59%, n = 41/70) and V (73%, n = 84/115), that clustered together as the CC1/alp3/PI-1+PI-2a genetic lineage, which has been mostly associated with serotype V. Some serotype Ib isolates were also present in CC12 (n = 25/48), defined as the CC12/bca/PI-1+PI-2a genetic lineage, which is the most frequent CC associated with serotype Ib. Throughout the study period there was a significant increase of CC1 (p (CA) = 0.027) and a decrease of CC12 (p (CA) < 0.001) (Figure 2). The increase of serotype Ib accompanied the increase of CC1 and the complementary decrease of CC12.

CC23 is knowingly associated with serotype Ia, and in this collection most serotype Ia isolates grouped within this CC (80%, n = 113/142). The presence of three sub-lineages within CC23 was evident, such as ST23/eps/PI-2a (n = 60/131), ST24/bca/ PI-2a (n = 25/131), ST144/rib/PI-2a (n = 11/131) and respective SLVs.

CC19 enclosed mostly serotypes II (n = 27/67) and III (n = 28/67), represented by ST28 and ST19, respectively, part of the genetic lineage CC19/rib/PI-1+PI-2a.

CC17 comprised the other half of the serotype III isolates (n = 23/55), defined by CC17/rib/PI-1+PI-2b, the hypervirulent clone often associated with neonatal

invasive disease (Jones et al., 2003; Manning et al., 2009; Martins et al., 2017, 2007).

A small number of serotype V isolates (10%, n = 12/115) was defined by the genetic lineage CC26/PI-2a, lacking the alp surface protein gene.

A small amount of serotype IX isolates (n = 6) grouped together as the CC130/bca/PI-2a genetic lineage.

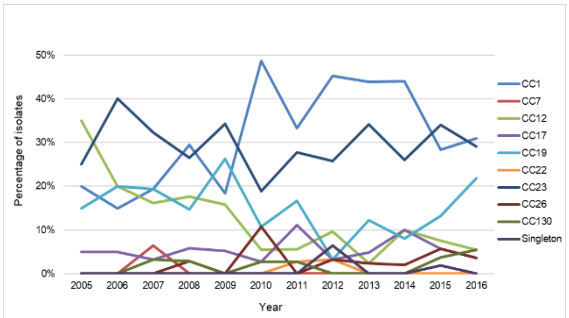

Figure 2 – CC distribution of the SSTI GBS isolates from 2005 to 2016

Antimicrobial Susceptibility Testing and Resistance Genotypes

All 446 GBS SSTI isolates were susceptible to penicillin, vancomycin and gentamicin. Chloramphenicol and levofloxacin resistance was found in 0.4% (n = 2) and 0.9% (n = 4) of the isolates, respectively. The overall rate of erythromycin resistance was 25% (n = 113) and clindamycin resistance was 23% (n = 103). From 2005 until 2016 macrolide and lincosamide resistance increased significantly (p (CA) < 0.001 and p (CA) = 0.001), respectively) (Figure 3). Macrolide and lincosamide resistance phenotypes were identified in 25.8% (n = 115) of the collection. Of these 115 resistant isolates, 66.1% (n = 76) presented the $cMLS_B$ phenotype, 21.8% (n = 25) the $iMLS_B$ phenotype, 10.4% (n = 12) the M phenotype and 1.7% (n = 2) the LS_A phenotype. Most of the isolates presenting the $cMLS_B$ phenotype carried the *erm*(B) gene (92%, n = 70/76), the $iMLS_B$ phenotype was mostly associated with the *erm*(TR) gene (96%, n = 24/25), and all the isolates presenting the M and LS_A phenotypes carried the *mef*(E) gene and the *lsa*(C) gene, respectively. The *lnu*(B) gene was not found in any isolate.

High-level resistance to streptomycin was found in 1.3% (n = 6) of the isolates, which possessed either the *aph(3')-III* gene or both the *aph(3')-III* and *ant(6)-Ia* genes. These GBS isolates were not only resistant to streptomycin, but were also resistant to erythromycin and clindamycin, presenting the $cMLS_B$ phenotype. The first isolate with these characteristics was detected in 2005, later other isolates were found in 2009 and 2014, and aside from the multidrug resistance mentioned, these GBS SSTI isolates were unlike one another in other aspects as they differed in serotype, surface protein, pilus islands, ST and CC.

Tetracycline resistance was present in 77% of the GBS SSTI isolates (n = 344) and was associated with different genes, mainly by the *tet*(M) gene, present in 95.1% of the GBS isolates (n = 327), but also 2.6% (n = 9) possessed the *tet*(O) gene and 1.4% (n = 5) possessed both the *tet*(M)+*tet*(O) genes. The *tet*(L) gene was only present in 1 isolate in association with the *tet*(M) gene. In 2 cases, although the isolates were phenotypically resistant, none of the genes tested were found.

There was an overrepresentation of the resistance phenotype $cMLS_B$ within CC1 ($p < 0.001$), particularly associated with the serotype Ib ($p < 0.001$), as 95% of the serotype Ib isolates within CC1 were $cMLS_B$ (n = 39/41). There was also a significant association between CC23 and serotype Ia to macrolide and lincosamide susceptibility ($p < 0.001$). Furthermore, the $iMLS_B$ phenotype was exclusively represented in serotypes III ($p < 0.001$) and V ($p = 0.016$).

The GBS SSTI isolates that presented the LS_A phenotype (n = 2) were serotype II and belonged to the CC19/rib/PI-1+PI-2a genetic lineage.

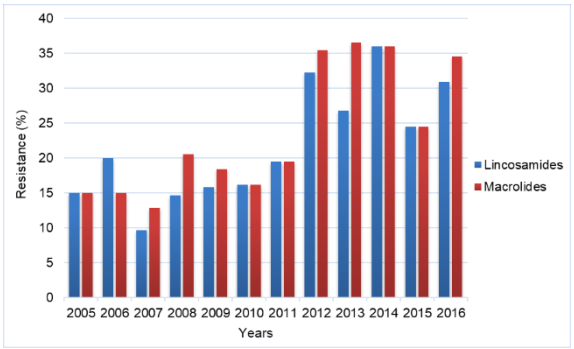

Figure 3 – Macrolide and lincosamide resistance rates of GBS SSTI isolates, from 2005 to 2016

Discussion

GBS is an important pathogen responsible for both adult and neonatal disease. Although increasing research has been done regarding GBS invasive disease in non-pregnant adults, not as much as been invested in the study of SSTI. Considering that SSTI is the most common manifestation of GBS infection in non-pregnant adults (Farley, 2001b; Le Doare and Heath, 2013; Schuchat and Balter, 2006; Skoff et al., 2009) it would be interesting to compare the GBS diversity found among invasive disease cases and SSTI in non-pregnant adults. The increase of GBS disease in adults is evident worldwide, particularly in the elderly population (Edwards et al., 2016; Farley, 2001; Skoff et al., 2009). In contrast, the incidence of GBS disease in newborns seems to be overall decreasing worldwide (Madrid et al., 2017), which might be related to the increasing awareness and use of prevention strategies, although different regions and countries may present different estimates. In this study it was evidenced an overall increase of SSTI in both elderly and young adults, however there was a higher frequency of SSTI among the elderly. This is similar to what was reported in the most recent study regarding GBS invasive disease in Portugal (Lopes et al., 2018), suggesting that independently of the GBS disease presentation, there is an overall increasing trend, in which the elderly seem to be particularly at risk. This is most likely due to underlying diseases, recognized as risk factors, such as diabetes, cardiovascular disease, and cancer, that are known to debilitate the immune system (Farley, 2001b; Skoff et al., 2009; Schuchat and Balter, 2006).

In the study collection substantial serotype diversity was observed. Similarly, in the most recent Portuguese publication regarding invasive GBS disease in non-pregnant adults, considerable serotype diversity was found, as expected the broad spectrum of disease presentations (Lopes et al., 2018). In Europe and North America, the serotypes responsible for the great majority of GBS invasive disease in non-pregnant adults are serotypes Ia, V and III, with some prevalence variations depending on the geographical location and time period (Lamagni et al., 2013; Skoff et al., 2009; Tazi et al., 2011; Teatero et al., 2014). In Portugal, in previous years, there was a similar serotype distribution with a higher prevalence of serotype Ia, followed by serotypes V and III (Martins et al., 2012). Nevertheless, a recent study has shown a clear change of the serotype distribution in Portugal, where serotype Ib has become the most frequent after 2013 (Lopes et al., 2018). The association of serotype Ib with invasive disease in adults is rather low in most countries, but a similar situation was reported in Japan, with serotype Ib being the most common among invasive disease cases in non-pregnant adults, followed by serotypes V and III (Morozumi et al., 2016).

Serotyping showed that serotypes Ia, V, Ib, III, and II were responsible for over 85% of SSTI in Portugal and overall serotype Ia was the most frequent, similarly to what was previously reported among invasive disease cases (Lopes et al., 2018). As the years progressed, there were significant changes in the serotype distribution, namely the decrease of serotype Ia and the increase of serotype Ib. This increase was more noticeable after 2011, and in 2016 serotype Ib became the most frequent serotype, being responsible for 25% of all SSTI (Figure 1), a similar trend to what was observed in invasive disease (Lopes et al., 2018). This shows that this serotype has emerged and is successfully established in Portugal as cause of both invasive disease and SSTI in non-pregnant adults.

The association between CCs and serotypes was evidenced upon the creation of the MLST database (Jones et al., 2003). A significant number of studies has shown associations between CC1 and serotype V, between CC12, grouping ST8, ST10 and ST12, and serotype Ib, and between CC23 and serotype Ia, regardless of the geographic location (Björnsdóttir et al., 2016; Jones et al., 2003; Meehan et al., 2014; Morozumi et al., 2016). Similarly to what was observed among invasive disease cases in Portugal (Lopes et al., 2018), serotype Ia was overall the most frequent serotype. On the other hand, CC23, which is usually associated with serotype Ia, was not the most frequent CC. Rather, in the study collection an unusual number of serotype Ib isolates associated with CC1 were found, contributing together with

serotype V, to the increasing prevalence of CC1. The association of serotype Ib and CC1 was also observed in the most recent study of invasive disease in non-pregnant adults in Portugal (Lopes et al., 2018).

The association between serotype Ib and CC1 is uncommon, and this new serotype/genotype combination is characterized by the presence of the surface protein gene alp3 and both PI-1 and PI-2a, which has been almost exclusively associated with serotype V. Recently a Canadian study showed through genomic analysis that the serotype Ib/ST1 lineage originated from a serotype V/ST1 strain which suffered horizontal transfer of the *cps* locus, known as capsular switching, replacing *cps*V for *cps*Ib (Neemuchwala et al., 2016). This capsular switching event created this novel genetic lineage: serotype Ib/CC1/alp3/PI-1+PI-2a. In Portugal this genetic lineage was first noticed in GBS neonatal infections (Martins et al., 2017), and later its presence was also noted in invasive disease cases in non-pregnant adults (Lopes et al., 2018), with serotype Ib being responsible for 35% of infections in 2015. The increasing frequency of this genetic lineage in Portugal appears to have started in 2011, becoming more prevalent in GBS disease in adults in recent years. However, the reasons for its significant expansion in Portugal, while this clone does not appear to be particularly predominant elsewhere, are still not clear. In Japan, most serotype Ib isolates were grouped within the characteristic CC12, although a small number of isolates clustered within CC1 (Morozumi et al., 2016). In Canada, few serotype Ib/ST1 isolates were identified (Neemuchwala et al., 2016) and recently in the USA, some serotype Ib/ST1 isolates were also found (Metcalf et al., 2017). This capsular switching event appears to be happening in countries other than Portugal, although its emergence is not as evident or predominant. The reason why this lineage appears to be so widespread and established in the Portuguese adult population might be due to some specific fitness characteristics which lead to its advantage, either in causing disease or colonizing asymptomatically when compared with other lineages, or due to the fact that some beneficial selective pressures may be acting upon this lineage (Lopes et al., 2018).

Serotype V was mainly grouped within CC1 and although serotype V frequency increased from 2005 to 2010, from then on until 2016 there was an evident decrease, contrasting with the rise of the serotype Ib/CC1 genetic lineage that took place in the same period. This is consistent with the capsular switching event above mentioned, suggesting that the new serotype/genotype combination may be replacing the serotype V/CC1 lineage.

In the study collection, 10% of serotype V isolates (n = 12) represented CC26/PI-2a with no surface protein gene detected. A small number of isolates with these characteristics was also found in GBS invasive disease in non-pregnant adults in Portugal (Lopes et al., 2018) and considering that in previous years this lineage was not identified, it suggests its recent introduction in Portugal. The serotype V/CC26 appears to be frequent in African countries (Brochet et al., 2009; Huber et al., 2011), and it is also present in a smaller proportion in Japan (Morozumi et al., 2016), but it remains infrequent in most European countries, with one isolate having been identified in both Poland (Sadowy et al., 2010) and Spain (Martins et al., 2011).

Within CC23, where the majority of serotype Ia isolates clustered together, three sub-lineages were present. The majority of CC23 isolates belonged to the ST23/eps/PI-2a genetic sub-lineage and its SLVs ST262, ST640 and ST1203, which is already known as the most common sub-lineage, being not only responsible for GBS invasive disease in adults, but also affecting neonates (Martins et al., 2017, 2012). There was also a significant number of isolates representing the genetic sub-lineage ST24/bca/PI-2a and its SLVs ST498 and ST707, which was previously identified as a successful clone within the geographical boundaries of the Mediterranean region, being found in Italy (Gherardi et al., 2007) and with a higher frequency in Spain (Martins et al., 2011) and Portugal (Martins et al., 2012). A smaller amount of serotype Ia/CC23 belonged to the ST144/rib/PI-2a genetic sub-lineage, which has been circulating for over a decade in Portugal with little expression (Martins et al., 2007). This lineage appears to be

infrequent in most countries, with one isolate found in both Iceland and Ireland (Björnsdóttir et al., 2016; Meehan et al., 2014).

In the study collection a small number of serotype IX isolates were identified, belonging to the CC130/ST130. This serotype was recently reported in Portugal (Martins et al., 2017), but it might have been circulating previously in the country unnoticed, given that the description of serotype IX is relatively recent (Creti et al, 2004), and the type IX sera was not yet commercially available. This might be the case for one NT isolate that belonged to ST130 in invasive disease in non-pregnant adults prior to 2008 (Martins et al., 2012). In the GBS SSTI isolates, serotype IX/CC130 lineage was exclusively responsible for infections in elderly adults, similarly to what was observed in GBS invasive disease in Portugal (Lopes et al., 2018), where a large proportion of isolates from this lineage were also associated with elderly adults. It was also found one isolate in neonatal invasive disease cases in Portugal (Martins et al., 2017). Serotype IX is quite rare in European countries, with few cases being identified throughout the years (Lamagni et al., 2013; Lambertsen et al., 2010; Meehan et al., 2014). Similarly to what has happened in Portugal, a higher number of cases may exist that were classified has NT. A small amount of serotype IX isolates were identified in Canada (Alhhazmi et al., 2016; Teatero et al., 2014), and were also associated with elderly adults. Thus, this serotype, although infrequent in most countries, seems to be established in Portugal, and appears to be highly associated with elderly people, independently of the GBS disease manifestation.

Erythromycin and clindamycin resistance rates (25% and 23%, respectively) increased throughout the study period. A significant association was found between serotype Ib and the macrolide resistance phenotype $cMLS_B$, present in 95% of the isolates. On the other hand, serotype Ia/CC23 isolates were associated with and macrolide and lincosamide susceptibility. Given the changes in serotype distribution in the study period, with the susceptible serotype Ia/CC23 decreasing and the serotype Ib/CC1 macrolide resistant lineage increasing, this expansion is likely the major driver of the increase of macrolide resistance in Portugal.

High-level resistance to streptomycin was found in 6 isolates, which also presented the $cMLS_B$ phenotype. In Portugal, streptomycin resistant isolates were already recently found not only in neonates (associated with the serotype III/ST17/PI-2b genetic lineage) (Martins et al., 2017) but also in invasive disease in non-pregnant adults (Lopes et al., 2018). High-level streptomycin resistant isolates were also found in China, associated with neonates (Campisi et al., 2016), in Canada, mostly linked to neonatal disease, and in Kuwait, related to both pregnant women and neonates (Boswihi et al., 2012), with these isolates being represented by the serotype III/ST17/PI-2b genetic lineage. In contrast, the isolates found in this study were associated with different serotypes and to various genetic lineages and the first isolate dated 2005, raising the hypothesis that the genetic determinants of resistance may be spreading across lineages for over a decade. The emergence of multidrug resistant isolates in multiple countries raises concern regarding the efficacy of therapeutic strategies to fight GBS infections.

This study complements the research previously done in Portugal regarding invasive disease in adults, providing an additional perspective of the lineages causing SSTI. In this study it was demonstrated the increase of SSTI over the years, parallel to the increase of GBS invasive disease (Lopes et al., 2018), with both studies showing a higher frequency of cases among the elderly. This study also showed the diversity of genetic lineages present in Portugal, including the recent introduction of relatively uncommon genetic lineages when comparing to other European and North American countries. Finally, this study also shows the emergence of the serotype Ib/CC1 genetic lineage, already known to have happened in GBS invasive disease, showing that the capsular switching event generating this new lineage resulted in a successful clone that is well established as leading cause of different GBS disease presentations in Portuguese adults. This change of the serotype distribution in Portugal is worrisome; the decrease of a macrolide and lincosamide susceptible

serotype Ia lineage is being contrasted with the increase of the macrolide and lincosamide resistant serotype Ib/CC1 genetic lineage. It is unclear why the serotype Ib/CC1 macrolide-resistant lineage is expanding so markedly in Portugal, while it appears to be widely disseminated in other countries but not particularly prevalent elsewhere.

References

Alhhazmi, A., Hurteau, D., Tyrrell, G.J., 2016. Epidemiology of Invasive Group B Streptococcal Disease in Alberta, Canada, from 2003 to 2013. J. Clin. Microbiol. 54, 1774–1781.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate – a practical and powerful approach to multiple testing. J. R. Stat. Soc. 57, 289–300.

Bergseng, H., Rygg, M., Bevanger, L., Bergh, K., 2008. Invasive group B streptococcus (GBS) disease in Norway 1996–2006. Eur. J. Clin. Microbiol. Infect. Dis. 27, 1193–1199.

Björnsdóttir, E.S., Martins, E.R., Erlendsdóttir, H., Haraldsson, G., Melo-Cristino, J., Kristinsson, K.G., Ramirez, M., 2016. Changing epidemiology of group B streptococcal infections among adults in Iceland: 1975–2014. Clin. Microbiol. Infect. 22, 379.e9-379.e16.

Boswihi, S.S., Udo, E.E., Al-Sweih, N., 2012. Serotypes and antibiotic resistance in Group B streptococcus isolated from patients at the Maternity Hospital, Kuwait. J. Med. Microbiol. 61, 126–131.

Bozdogan, B.L., Berrezouga, L., Kuo, M.-S., Yurek, D.A., Farley, K.A., Stockman, B.J., Leclercq, R., 1999. A New Resistance Gene, linB, Conferring Resistance to Lincosamides by Nucleotidylation in Enterococcus faecium HM1025. Antimicrob. Agents Chemother. 43, 925–929.

Brochet, M., Couve, E., Bercion, R., Sire, J.-M., Glaser, P., 2009. Population Structure of Human Isolates of Streptococcus agalactiae from Dakar and Bangui. J. Clin. Microbiol. 47, 800–803.

Campisi, E., Rosini, R., Ji, W., Guidotti, S., Rojas-López, M., Geng, G., Deng, Q., Zhong, H., Wang, W., Liu, H., Nan, C., Margarit, I., Rinaudo, C.D., 2016. Genomic Analysis Reveals Multi-Drug Resistance Clusters in Group B Streptococcus CC17 Hypervirulent Isolates Causing Neonatal Invasive Disease in Southern Mainland China. Front. Microbiol. 7, 1–11.

Carrico, J.A., Silva-Costa, C., Melo-Cristino, J., Pinto, F.R., de Lencastre, H., Almeida, J.S., Ramirez, M., 2006. Illustration of a Common Framework for Relating Multiple Typing Methods by Application to Macrolide-Resistant Streptococcus pyogenes. J. Clin. Microbiol. 44, 2524–2532.

Castor, M.L., Whitney, C.G., Como-Sabetti, K., Facklam, R.R., Ferrieri, P., Bartkus, J.M., Juni, B.A., Cieslak, P.R., Farley, M.M., Dumas, N.B., Schrag, S.J., Lynfield, R., 2008. Antibiotic Resistance Patterns in Invasive Group B Streptococcal Isolates. Infect. Dis. Obstet. Gynecol. 2008, 1–5.

Clark, N.C., Olsvik, Ø., Swenson, J.M., Spiegel, C.A., Tenover, F.C., 1999. Detection of a Streptomycin/Spectinomycin Adenylyltransferase Gene (aadA) in Enterococcus faecalis. Antimicrob. Agents Chemother. 43, 157–160.

CLSI, 2015. Clinical and Laboratory Standards Institute - Performance Standards for Antimicrobial Susceptibility Testing; 240.

Compain, F., Hays, C., Touak, G., Dmytruk, N., Trieu-Cuot, P., Joubrel, C., Poyart, C., 2014. Molecular Characterization of Streptococcus agalactiae Isolates Harboring Small *erm* (T)-Carrying Plasmids. Antimicrob. Agents Chemother. 58, 6928–6930.

Creti, R., Fabretti, F., Orefici, G., von Hunolstein, C., 2004. Multiplex PCR Assay for Direct Identification of Group B Streptococcal Alpha-Protein-Like Protein Genes. J. Clin. Microbiol. 42, 1326–1329.

Edwards, M.S., Rench, M.A., Rinaudo, C.D., Fabbrini, M., Tuscano, G., Buffi, G., Bartolini, E., Bonacci, S., Baker, C.J., Margarit, I., 2016. Immune Response to Invasive Group B *Streptococcus* Disease in Adults. Emerg. Infect. Dis. 22, 1877–1883.

Farley, M.M., 2001. Group B Streptococcal Disease in Nonpregnant Adults. Clin. Infect. Dis. 33, 556–561.

Figueira-Coelho, J., Ramirez, M., Salgado, M.J., Melo-Cristino, J., 2004. *Streptococcus agalactiae* in a Large Portuguese Teaching Hospital: Antimicrobial Susceptibility, Serotype Distribution, and Clonal Analysis of Macrolide-Resistant Isolates. Microb. Drug Resist. 10, 31–36.

Gherardi, G., Imperi, M., Baldassarri, L., Pataracchia, M., Alfarone, G., Recchia, S., Orefici, G., Dicuonzo, G., Creti, R., 2007. Molecular Epidemiology and Distribution of Serotypes, Surface Proteins, and Antibiotic Resistance among Group B Streptococci in Italy. J. Clin. Microbiol. 45, 2909–2916.

Huber, C.A., McOdimba, F., Pflueger, V., Daubenberger, C.A., Revathi, G., 2011. Characterization of Invasive and Colonizing Isolates of Streptococcus agalactiae in East African Adults. J. Clin. Microbiol. 49, 3652–3655.

Jones, N., Bohnsack, J.F., Takahashi, S., Oliver, K.A., Chan, M.-S., Kunst, F., Glaser, P., Rusniok, C., Crook, D.W.M., Harding, R.M., Bisharat, N., Spratt, B.G., 2003. Multilocus Sequence Typing System for Group B Streptococcus. J. Clin. Microbiol. 41, 2530–2536.

Kimura, K., Suzuki, S., Wachino, J. -i., Kurokawa, H., Yamane, K., Shibata, N., Nagano, N., Kato, H., Shibayama, K., Arakawa, Y., 2008. First Molecular Characterization of Group B Streptococci with Reduced Penicillin Susceptibility. Antimicrob. Agents Chemother. 52, 2890–2897.

Kobayashi, M., Schrag, S.J., Alderson, M.R., Madhi, S.A., Baker, C.J., Sobanjo-ter Meulen, A., Kaslow, D.C., Smith, P.G., Moorthy, V.S., Vekemans, J., 2016. WHO consultation on group B Streptococcus vaccine development: Report from a meeting held on 27–28 April 2016. Vaccine.

Lamagni, T.L., Keshishian, C., Efstratiou, A., Guy, R., Henderson, K.L., Broughton, K., Sheridan, E., 2013. Emerging Trends in the Epidemiology of Invasive Group B Streptococcal Disease in England and Wales, 1991–2010. Clin. Infect. Dis. 57, 682–688.

Lambertsen, L., Ekelund, K., Skovsted, I.C., Liboriussen, A., Slotved, H.-C., 2010. Characterisation of invasive group B streptococci from adults in Denmark 1999 to 2004. Eur. J. Clin. Microbiol. Infect. Dis. 29, 1071–1077.

Le Doare, K., Heath, P.T., 2013. An overview of global GBS epidemiology. Vaccine 31, D7–D12.

Lopes, E., Fernandes, T., Machado, M.P., Carriço, J.A., Melo-Cristino, J., Ramirez, M., Martins, E.R., the Portuguese Group for the Study of Streptococcal Infections, 2018. Increasing macrolide resistance among Streptococcus agalactiae causing invasive disease in non-pregnant adults was driven by a single capsular-

transformed lineage, Portugal, 2009 to 2015. Eurosurveillance 23, 1–10.

Madrid, L., Seale, A.C., Kohli-Lynch, M., Edmond, K.M., Lawn, J.E., Heath, P.T., Madhi, S.A., Baker, C.J., Bartlett, L., Cutland, C., Gravett, M.G., Ip, M., Le Doare, K., Rubens, C.E., Saha, S.K., Sobanjo-ter Meulen, A., Vekemans, J., Schrag, S., for the Infant GBS Disease Investigator Group, Agarwal, R., da Silva, A.R.A., Bassat, Q., Berkley, J.A., Dangor, Z., Dhaded, S., Giannoni, E., Hammoud, M., Kobayahsi, M., O'Sullivan, C., Sakata, H., Sridhar, S., Sigaúque, B., Tyrrell, G., Paul, V., 2017. Infant Group B Streptococcal Disease Incidence and Serotypes Worldwide: Systematic Review and Meta-analyses. Clin. Infect. Dis. 65, S160–S172.

Malbruny, B., Werno, A.M., Murdoch, D.R., Leclercq, R., Cattoir, V., 2011. Cross-Resistance to Lincosamides, Streptogramins A, and Pleuromutilins Due to the *lsa* (C) Gene in *Streptococcus agalactiae* UCN70. Antimicrob. Agents Chemother. 55, 1470–1474.

Manning, S.D., 2014. Emergence of a hypervirulent neonatal pathogen. Lancet Infect. Dis. 14, 1028–1030.

Martins, E.R., Andreu, A., Correia, P., Juncosa, T., Bosch, J., Ramirez, M., Melo-Cristino, J., on behalf of the Microbiologist Group for the Study of Vertical Transmission Infections from the Catalan Society for Clinical Microbiology and Infectious Diseases, 2011. Group B Streptococci Causing Neonatal Infections in Barcelona Are a Stable Clonal Population: 18-Year Surveillance. J. Clin. Microbiol. 49, 2911–2918.

Martins, E.R., Melo-Cristino, J., Ramirez, M., 2010. Evidence for Rare Capsular Switching in Streptococcus agalactiae. J. Bacteriol. 192, 1361–1369.

Martins, E.R., Melo-Cristino, J., Ramirez, M., 2007. Reevaluating the Serotype II Capsular Locus of Streptococcus agalactiae. J. Clin. Microbiol. 45, 3384–3386.

Martins, E.R., Melo-Cristino, J., Ramirez, M., the Portuguese Group for the Study of Streptococcal Infections, 2012. Dominance of Serotype Ia among Group B Streptococci Causing Invasive Infections in Nonpregnant Adults in Portugal. J. Clin. Microbiol. 50, 1219–1227.

Martins, E.R., Pedroso-Roussado, C., Melo-Cristino, J., Ramirez, M., The Portuguese Group for the Study of Streptococcal Infections, 2017. Streptococcus agalactiae Causing Neonatal Infections in Portugal (2005–2015): Diversification and Emergence of a CC17/PI-2b Multidrug Resistant Sublineage. Front. Microbiol. 8, 1–9.

Meehan, M., Cunney, R., Cafferkey, M., 2014. Molecular epidemiology of group B streptococci in Ireland reveals a diverse population with evidence of capsular switching. Eur. J. Clin. Microbiol. Infect. Dis. 33, 1155–1162.

Metcalf, B.J., Chochua, S., Gertz, R.E., Hawkins, P.A., Ricaldi, J., Li, Z., Walker, H., Tran, T., Rivers, J., Mathis, S., Jackson, D., Glennen, A., Lynfield, R., McGee, L., Beall, B., 2017. Short-read whole genome sequencing for determination of antimicrobial resistance mechanisms and capsular serotypes of current invasive Streptococcus agalactiae recovered in the USA. Clin. Microbiol. Infect. 23, 574.e7-574.e14.

Morozumi, M., Wajima, T., Takata, M., Iwata, S., Ubukata, K., 2016. Molecular Characteristics of Group B Streptococci Isolated from Adults with Invasive Infections in Japan. J. Clin. Microbiol. 54, 2695–2700.

Nascimento, M., Sousa, A., Ramirez, M., Francisco, A.P., Carriço, J.A., Vaz, C., 2017. PHYLOViZ 2.0: providing scalable data integration and visualization for multiple phylogenetic inference methods. Bioinformatics 33, 128–129.

Neemuchwala, A., Teatero, S., Athey, T.B.T., McGeer, A., Fittipaldi, N., 2016. Capsular Switching and Other Large-Scale Recombination Events in Invasive Sequence Type 1 Group B *Streptococcus*. Emerg. Infect. Dis. 22, 1941–1944.

Phares, C.R., Lynfield, R., Farley, M.M., Mohle-Boetani, J., Harrison, L.H., Petit, S., Craig, A.S., Schaffner, W., Zansky, S.M., Gershman, K., Stefonek, K.R., Albanese, B.A., Zell, E.R., Schuchat, A., Schrag, S.J., 2008. Epidemiology of Invasive Group B Streptococcal Disease in the United States,. JAMA 299, 10.

Sadowy, E., Matynia, B., Hryniewicz, W., 2010. Population structure, virulence factors and resistance determinants of invasive, non-invasive and colonizing Streptococcus agalactiae in Poland. J. Antimicrob. Chemother. 65, 1907–1914.

Schuchat, A., 1998. Epidemiology of Group B Streptococcal Disease in the United States: Shifting Paradigms. CLIN MICROBIOL REV 11, 1–17.

Schuchat, A., Balter, S., 2006. Epidemiology of Group B Streptococcal Infections, in: Rood, J.I., Ferretti, J.J., Fischetti, V.A., Portnoy, D.A., Novick, R.P. (Eds.), Gram-Positive Pathogens, Second Edition. American Society of Microbiology, pp. 186–195.

Skoff, T.H., Farley, M.M., Petit, S., Craig, A.S., Schaffner, W., Gershman, K., Harrison, L.H., Lynfield, R., Mohle-Boetani, J., Zansky, S., Albanese, B.A., Stefonek, K., Zell, E.R., Jackson, D., Thompson, T., Schrag, S.J., 2009. Increasing Burden of Invasive Group B Streptococcal Disease in Nonpregnant Adults, 1990–2007. Clin. Infect. Dis. 49, 85–92.

Tazi, A., Morand, P.C., Réglier-Poupet, H., Dmytruk, N., Billoët, A., Antona, D., Trieu-Cuot, P., Poyart, C., 2011. Invasive group B streptococcal infections in adults, France (2007–2010). Clin. Microbiol. Infect. 17, 1587–1589.

Teatero, S., McGeer, A., Low, D.E., Li, A., Demczuk, W., Martin, I., Fittipaldi, N., 2014. Characterization of Invasive Group B Streptococcus Strains from the Greater Toronto Area, Canada. J. Clin. Microbiol. 52, 1441–1447.

Trzcinski, K., Cooper, B.S., Hryniewicz, W., Dowson, C.G., 2000. Expression of resistance to tetracyclines in strains of methicillin-resistant Staphylococcus aureus. J. Antimicrob. Chemother. 45, 763–770.

Vakulenko, S.B., Donabedian, S.M., Voskresenskiy, A.M., Zervos, M.J., Lerner, S.A., Chow, J.W., 2003. Multiplex PCR for Detection of Aminoglycoside Resistance Genes in Enterococci. Antimicrob. Agents Chemother. 47, 1423–1426.

Wang, Y.-H., Chen, H.-M., Yang, Y.-H., Yang, T.-H., Teng, C.-H., Chen, C.-L., Chu, C., Chiu, C.-H., 2014. Clinical and microbiological characteristics of recurrent group B streptococcal infection among non-pregnant adults. Int. J. Infect. Dis. 26, 140–145.