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

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“Once we accept our limits, we go beyond them”

Albert Einstein

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Resumo

No período de 2005 a 2016, em Portugal, foram isolados *Streptococcus* do Grupo B (SGB) responsáveis por infeções da pele e tecidos moles em adultos, excluindo grávidas. As estirpes de SGB (n = 446) foram caracterizadas por serotipagem e determinação dos perfis de proteínas de superfície e das ilhas genómicas que codificam os pili. Foram realizados testes de suscetibilidade a antimicrobianos e foram identificados, através de PCR, os genes de resistência. Os *sequence types* (ST) e complexos clonais (CC) foram atribuídos a cada estirpe por *multilocus sequence typing* (MLST). O serotipo Ia foi o mais frequente (31,8%), seguido do serotipo V (25,8%) e do serotipo Ib (15,7%). No período em estudo observou-se um aumento significativo do serotipo Ib (p (CA) = 0.018), acompanhado de uma diminuição do serotipo Ia (p (CA) = 0.032). Em 2016 o serotipo Ib passou a ser o mais frequente, sendo responsável por 25% das infeções da pele e tecidos moles. De todas as estirpes serotipo Ib, 59% foram agrupadas no CC1 (n = 41/70), sendo este o CC dominante, incluindo a maioria das estirpes do serotipo Ib e 73% das estirpes do serotipo V (n = 84/115). Estas estirpes fazem parte da linhagem genética CC1/*alp3*/PI-1+PI-2a, quase exclusivamente associada ao serotipo V. Esta nova combinação de serotipo/genótipo resultou de um evento de transformação capsular. Nesta recente linhagem genética verificou-se uma sobrerrepresentação do fenótipo de resistência $cMLS_B$ (p (CA) < 0.001), presente em 95% das estirpes serotipo Ib/CC1 (n = 39/41). Foram identificadas 6 estirpes que possuíam não só resistência de alto nível a estreptomicina, mas que também apresentavam o fenótipo de resistência $cMLS_B$. O aumento de uma linhagem genética altamente resistente a macrólidos e lincosamidas, bem como a identificação de estirpes multirresistentes a antibióticos, responsáveis por infeções da pele e tecidos moles em adultos em Portugal é preocupante. Estes dados evidenciam a importância da epidemiologia de SGB na monotorização da resistência a antimicrobianos das infeções por SGB.

Palavras chave: *Streptococcus* do Grupo B; Infeções da pele e tecidos moles; Adultos excluindo grávidas; Epidemiologia molecular; Linhagens genéticas; Resistência antimicrobiana; Resistência de alto nível à estreptomicina.

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 infection; Non-pregnant adults; Molecular epidemiology; Genetic lineage; Antimicrobial resistance; High-level streptomycin resistance.

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List of Abbreviations

GBS – Group B *Streptococcus*
EOD – Early-onset disease
LOD – Late-onset disease
USA – United States of America
IAP – Intrapartum antibiotic prophylaxis
SSTI – Skin and soft tissue infection
CPS – Capsular polysaccharide
ACP – Alpha C protein
BCP – Beta C protein
DNA – Deoxyribonucleic acid
Ig – Immunoglobulin
Alp – Alpha-like protein
PI – Pilus island
AP1 – Ancillary protein 1
AP2 – Ancillary protein 2
CRM197 – Non-toxic mutant of diphtheria toxin
PBP – Penicillin binding protein
MIC – Minimum inhibitory concentration
MLS_B – Macrolide-lincosamide-streptogramin B resistance phenotype
M – Macrolide resistance phenotype
LS_A – Lincosamide and streptogramin A resistance phenotype
tRNA – Transfer ribonucleic acid
mRNA – Messenger ribonucleic acid
QRDR – Quinolone resistance determining regions
cat – Chloramphenicol acetyltransferase
HLAR – High-level aminoglycoside resistance
NT – Non-typeable
AST – Antimicrobial susceptibility testing
CLSI – Clinical and Laboratory Standards Institute
EUCAST – The European Committee on Antimicrobial Susceptibility Testing
PCR – Polimerase chain reaction
MLST – Multilocus sequence typing
bp – Base pairs
ST – Sequence type
CC – Clonal complex
SLV – Single-locus variants
DLV – Double-locus variants
TLV – Triple-locus variants

WGS – Whole genome sequencing

Mbp – Mega base pairs

SID – Simpson's Index of Diversity

FDR – False Discovery Rate

CA – Cochran-Armitage

INE – Instituto Nacional de Estatística

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1. Introduction

1.1. General Overview

Streptococcus agalactiae is a Gram-positive, β-hemolytic, chain-forming cocci bacterium that was first identified in 1896 by Lehmann and Neumann (Lehmann and Neumann, 1896). In 1933, Rebecca Lancefield published a paper concerning the serological differentiation of β-hemolytic streptococci, based on the carbohydrate composition of bacterial antigens found on the cell wall, in which *S. agalactiae* was attributed to Group B, and as its only member, was henceforth also known as Group B *Streptococcus* (GBS) (Lancefield, 1933). At that time, GBS was not associated with human disease, it was instead a well-known cause of bovine mastitis (Lancefield, 1933). In 1935, Lancefield demonstrated the presence of a variety of β-hemolytic streptococci in the birth canal, including GBS, although it was not associated with severe disease (Lancefield, 1935). It was only in 1938 that the first report of fatal infections by GBS in humans appeared (Fry, 1938), still, only in the 1960s was GBS considered a relevant human pathogen and given significance after several reports on the presence of GBS among newborns with sepsis and meningitis (Paoletti et al., 2006). Later, in the 1980s and 1990s, GBS was associated with causing invasive disease in non-pregnant adults, where several studies demonstrated the severity of GBS infections, accompanied with a higher than predicted mortality rate, mostly in the elderly and immunocompromised (Schuchat, 1998). Nowadays, GBS is known as a colonizing agent and as a pathogen of both newborns and adults.

1.2. Colonization and Transmission

The overall worldwide prevalence of healthy women colonized with GBS in the genitourinary and gastrointestinal tracts is 15%, an estimated value given the different prevalence in various regions and countries (Russell et al., 2017). The gastrointestinal tract is the primary reservoir for GBS, but given its close proximity to the genital tract, GBS transference from intestinal flora is likely to occur (Vornhagen et al., 2017). GBS is thought to be transmitted person-to-person via multiple routes, the most well-known is vertical transmission, either during pregnancy, due to an ascending infection, or during birth, when the newborn aspirates contaminated amniotic/vaginal fluids (Figure 1) (Rajagopal, 2009; Vornhagen et al., 2017). Sexual and fecal-oral transmission have also been reported (Manning et al., 2004; Vornhagen et al., 2017).

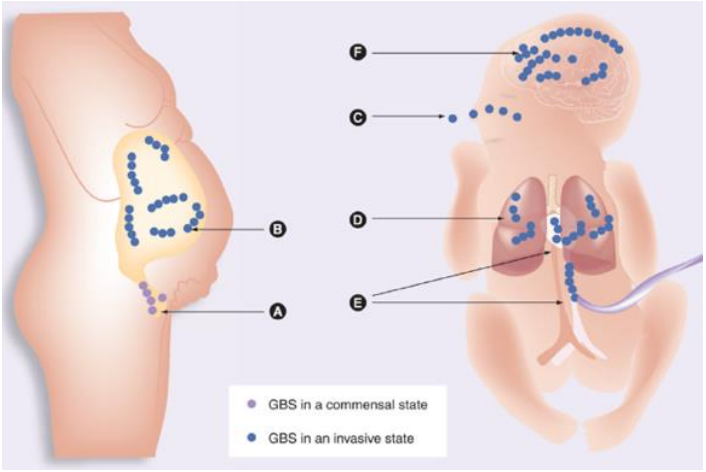

Figure 1 – Transmission route of GBS. (A) GBS colonizes genitourinary and gastrointestinal tracts. (B) GBS penetrates the gestational tissues. (C) Newborn aspirates GBS in utero or during birth. (D) GBS invades the neonatal lung causing pneumonia. (E) From the lung, GBS gains access into the bloodstream causing sepsis and invades multiple neonatal organs. (F) GBS penetrates the blood–brain barrier causing meningitis. Adapted from (Rajagopal, 2009).

1.3. Infections

1.3.1. Infection in Newborns

GBS infection in newborns usually presents as bacteremia, meningitis or pneumonia. Depending on the age of the newborn at disease onset, there are two different designations: early-onset disease (EOD), when it happens within the first week of life, usually within the first day, and late-onset disease (LOD) when the infection develops between one week and the first three months of life (Schuchat and Balter, 2006; Verani et al., 2010).

EOD happens due to the exposure of the newborn to GBS, present in the genitourinary tract of a colonized woman, either during the pregnancy or birth. When the newborn passes through the birth canal, he might aspirate contaminated amniotic/vaginal fluids leading to GBS colonization of the gastrointestinal tract and/or respiratory tract. GBS may also ascend from the vagina to the amniotic fluid after rupture of membranes, and the newborn aspirates contaminated amniotic fluid. This can also happen during pregnancy, as GBS can invade through intact membranes. When GBS is aspirated through the respiratory tract into the fetal lungs it might cause pneumonia. From the lung, GBS can gain access to the blood, which can lead to bacteremia (Schuchat and Balter, 2006; Verani et al., 2010; Vornhagen et al., 2017). EOD manifests mainly as respiratory failure and pneumonia, that rapidly progresses into bacteremia and sepsis. Although less common, meningitis and bone and soft tissue infection can also occur (Rajagopal, 2009; Schuchat and Balter, 2006). Maternal colonization with GBS is the most significant risk factor and a pre-requisite for EOD, as infants born to heavily colonized mothers are more likely to develop invasive GBS disease than infants born to mothers who are lightly colonized or not colonized at all. Other factors that increase the risk for developing EOD are gestational

age under 37 weeks, prolonged rupture of membranes, intra-amniotic infection and young maternal age (Schuchat and Balter, 2006; Verani et al., 2010).

In the case of LOD, GBS transmission is not completely understood, but it is thought that GBS colonization of the newborn might also happen by vertical transmission or due to nosocomial and horizontal transmission (Schuchat and Balter, 2006). LOD is frequently characterized by a bloodstream infection, with a high risk of development to meningitis, although it can also present as bone and soft tissue infections, urinary tract infections, or pneumonia (Rajagopal, 2009; Schuchat and Balter, 2006). LOD risk factors are not so well characterized, but recognized risk factors are vertical transmission, nosocomial acquisition, prematurity and young maternal age (Rajagopal, 2009; Schuchat, 1998; Schuchat and Balter, 2006).

GBS is a leading cause of morbidity and mortality among newborns worldwide, but the incidence of invasive GBS disease in newborns until three months of life varies worldwide, depending on the region and even between countries in the same region (Madrid et al., 2017). In 2012 a systematic review was published reporting an estimated overall incidence of GBS invasive disease in newborns: 0.53 per 1000 live births (Edmond et al., 2012). In 2017 an updated systematic review was published reporting a slightly lower estimated overall incidence of GBS disease in newborns: 0.49 per 1000 live births (Madrid et al., 2017). In the 2012 review, the estimated overall case-fatality rates for GBS infection in newborns was 9.6% and in 2017 a slight decrease to 8.4% (Edmond et al., 2012; Madrid et al., 2017).

1.3.2. Infection in Pregnant Women

GBS colonization of the genital tract during pregnancy is correlated with ascending infection during pregnancy, preterm birth, stillbirth, and neonatal infection (Vornhagen et al., 2017)[6].

Ascending infection is the route by which vaginal bacteria penetrate the cervical barrier, enter the uterus and penetrate gestational tissues (Figure 2). Once in the uterine space, GBS is present in the amniotic cavity or in contact with the placenta, where it causes multiple physiological events, potentially chorioamnionitis or inflammation of the placental membranes, chorioamniotic membrane rupture, cervical ripening, and uterine contraction, which are frequently linked with preterm births and still-births (Vornhagen et al., 2017, 2016). GBS may also cause a multitude of perinatal and postpartum infections, comprising both symptomatic and asymptomatic bacteriuria, endometritis, amnionitis, meningitis, pyelonephritis, and postpartum wound infections (Schuchat and Balter, 2006).

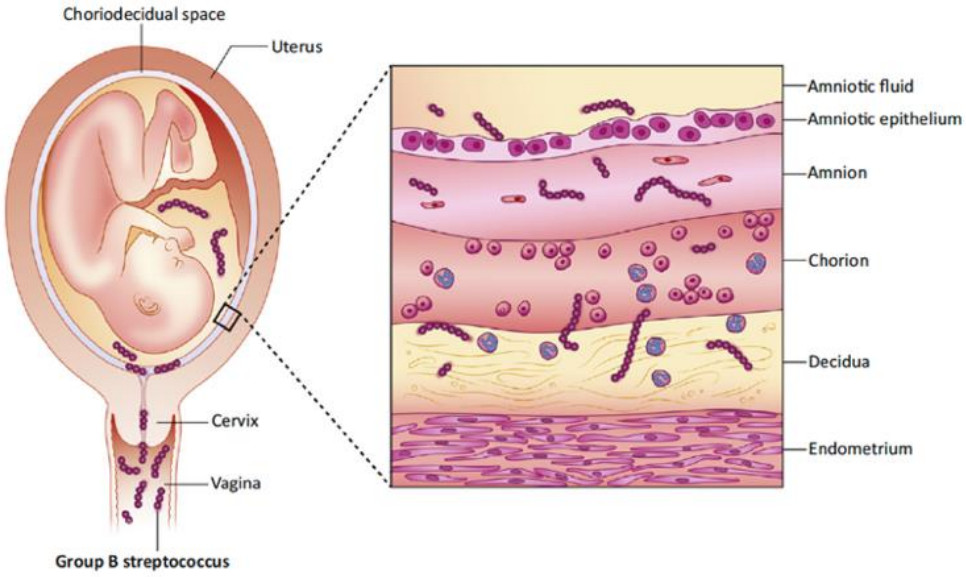

Figure 2 – Ascending infection by GBS. GBS vaginal colonization may result in the ascension of GBS from the vagina, through the cervix into the uterus, ultimately leading to bacterial invasion of placental membranes, the amniotic cavity, and the fetus. Adapted from (Vornhagen et al., 2017).

1.3.2.1. Prevention Strategies and Outcome

In the 1980s clinical trials demonstrated that giving intrapartum intravenous ampicillin or penicillin to pregnant women at risk for transmitting GBS to their newborn was highly effective at preventing invasive EOD. In the United States of America (USA), in 1996, guidelines for the prevention of perinatal GBS disease were issued recommending the use of a risk-based (women during birth who present fever, prolonged rupture of the membranes, or imminent preterm delivery) or screening-based approach (all women are screened for carriage of GBS between 35 and 37 weeks of gestation) to identify proper candidates for IAP (Centers for Disease Control and Prevention, 1996). In 2002 the guidelines were reviewed and updated for the recommendation of universal prenatal culture-based screening for vaginal and rectal GBS colonization of all pregnant women at 35-37 weeks' gestation (Centers for Disease Control and Prevention, 2002). After the implementation of these guidelines, several studies were published demonstrating a steep reduction of the incidence of EOD but no significant changes were observed in the incidence of LOD (Figure 3) (Centers for Disease Control and Prevention, 2007; Phares et al., 2008). In 2010, the guidelines were updated regarding the laboratory processing and management of pregnant women and newborns, to ensure early identification and treatment of early-onset GBS disease (Schrag and Verani, 2013; Verani et al., 2010). IAP was considered optimal if administered for at least ≥4 hours before delivery, consisting of intravenous penicillin, or ampicillin, and, for penicillin-allergic women clindamycin or vancomycin is recommended (Verani et al., 2010).

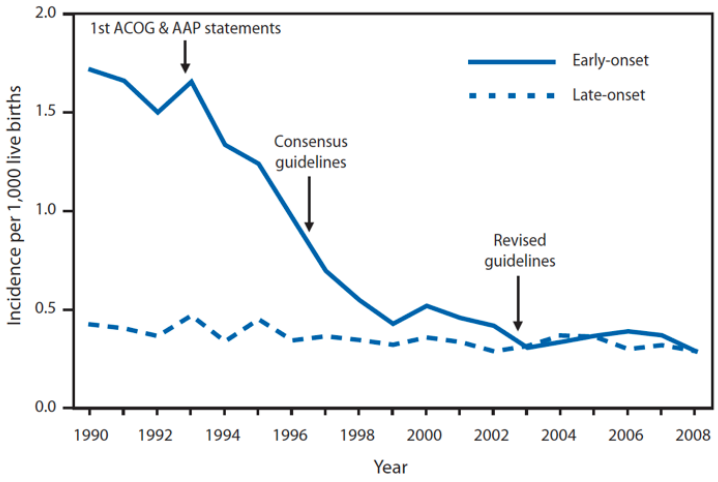

Figure 3 – Incidence of EOD and LOD from 1990-2008 in the USA. Adapted from (Verani et al., 2010).

Most European countries did not have nationwide or local protocols for surveillance and prevention of neonatal GBS disease. In 1999, only four of twenty-nine countries had nationwide guidelines for prevention of EOD (Trijbels-Smeulders et al., 2004). Eventually, countries started to set up prevention protocols, with most countries reporting decreasing incidences of EOD (de la Rosa Fraile et al., 2001).

In Portugal, it was only in 2004 that a protocol for screening and prevention of GBS perinatal disease was issued (Almeida et al., 2004). The protocol recommended universal screening of pregnant women at 35-37 weeks' gestation and the administration IAP to GBS carriers (Almeida et al., 2004). The rates of both case-fatality and incidence of GBS neonatal disease decreased nearly 40% (Neto, 2007). In 2013, national health authorities formally implemented universal GBS screening of pregnant women (DGS, Norma 37/2011, 2013).

These measures have contributed to the reduction of the high morbidity and case-fatality rates associated with EOD while also preventing invasive disease in pregnant women. Nevertheless, these approaches have not fully eliminated neonatal GBS infections, as the prevention strategies do not prevent LOD (Vornhagen et al., 2017). Overall, prevention of GBS infections in pregnant women and newborns is challenging and is affected by many factors such as pathogenicity of the GBS strain, host factors, changes in GBS antibiotic resistance (Vornhagen et al., 2017). As for now, penicillin is used as the first line agent for prevention and treatment of GBS disease, and for those allergic, clindamycin and vancomycin are considered suitable alternatives (Verani et al., 2010). GBS is mostly considered uniformly susceptible to β-lactams, although some isolates with reduced penicillin susceptibility were evidenced (Dahesh et al., 2008; Kimura et al., 2008; Verani et al., 2010). There is also the concern of increasing resistance to erythromycin and clindamycin in both neonatal and adult invasive infections, which have been reported worldwide in recent years, which suggests that the current prophylactic and therapeutic strategies are not a long-term solution (Castor et al., 2008; Lamagni et al., 2013; Martins et al., 2017). The development of a vaccine is the most promising approach to fight GBS disease.

1.3.3. Infection in Non-Pregnant Adults

GBS is a recognized and increasing cause of disease in non-pregnant adults, and although invasive and non-invasive GBS disease occurs in otherwise healthy adults, the majority of disease occurs amongst the elderly, particularly among those with significant underlying medical conditions (Farley, 2001a; Le Doare and Heath, 2013; Morozumi et al., 2016). The case fatality rate is higher in adults in comparison to neonates, due to the prevention strategies, and adults account for 90% of deaths by GBS infection in the USA (Edwards et al., 2016; Le Doare and Heath, 2013).

Regarding the incidence rate of GBS disease in non-pregnant adults, a significant increase in the USA was evident, between 1999 to 2007, from 3.6 cases/100.000 population to 7.3 cases/100.000 population, in ages 15-64 years. An even more remarkable increase was present among the elderly, from 21.5 cases/100.000 population to 26.0 cases/100.000, in the same time period (Le Doare and Heath, 2013; Skoff et al., 2009). In Canada (Alhhazmi et al., 2016), Japan (Morozumi et al., 2016) and several European countries (Bergseng et al., 2008; Lamagni et al., 2013; Lopes et al., 2018; Tazi et al., 2011) the same overall increasing trend has been reported, also being more noticeable in the elderly.

GBS infections in non-pregnant adults can be nosocomial or community-acquired and there are several clinical manifestations of GBS disease. The most common presentations of GBS infection include skin and soft-tissue infections (SSTI) as the leading clinical manifestation (which include cellulitis, ulcers and abscesses), bacteremia without focus, pneumonia and bone and joint infections. Less common presentations of GBS infection comprise arthritis, urinary tract infections, and peritonitis, as well as more severe clinical syndromes, such as meningitis and endocarditis, which are rare, but often associated with significant morbidity and mortality (Farley, 2001a; Le Doare and Heath, 2013; Schuchat and Balter, 2006; Skoff et al., 2009).

While ageing of the human population is a contributing factor to the increasing number and severity of invasive GBS disease cases among non-pregnant adults, risk factors other than age have been identified, namely underlying medical conditions, such as diabetes mellitus, cancer, heart, liver and neurological disease, and other forms of immunosuppressive conditions (Schuchat and Balter, 2006).

Diabetes is a predisposing condition to SSTI, which reflects the overrepresentation of diabetes among adults with invasive GBS disease (Edwards et al., 2016; Morozumi et al., 2016; Schuchat and Balter, 2006; Skoff et al., 2009). Although adults with diabetes do not present higher GBS colonization rates when compared with those without diabetes, there is evidence that abnormalities in immune function, such as neutrophil phagocytosis or intracellular killing may contribute to an increased risk of GBS disease in diabetic patients (Schuchat and Balter, 2006).

1.4. Importance of the Virulence Factors

Virulence factors are key components in infection and GBS encodes many virulence factors. GBS is a known colonizer of the genitourinary and gastrointestinal tracts, yet under certain circumstances it displays the ability to invade a variety of host tissues, evading immune detection and causing invasive disease (Maisey et al., 2008a; Rajagopal, 2009; Sendi et al., 2008). Throughout the process of colonization, GBS resides as a commensal organism and adapts to its host, however, GBS might transit into an invasive state, infiltrating different host niches such as the intrauterine compartment, neonatal lung and multiple organs, including the brain (Rajagopal, 2009; Sendi et al., 2008). The proper expression of GBS virulence factors, in response to the host environment provides a remarkable survival advantage to GBS (Rajagopal, 2009).

It is also important to understand virulence factors for their potential as vaccine targets. Immunity to GBS infections is elicited by the polysaccharide capsule and different cell surface proteins that possess protective antigens, making them promising vaccine targets (Paoletti et al., 2006; Wästfelt et al., 1996).

Table 1 summarizes some of the main virulence factors of GBS, describing the mode of action of the different pathogenic mechanisms, essential for its ability to cause disease. Those that contribute to the epidemiological characterization of GBS isolates in this study will be further elaborated.

Table 1 – Main virulence factors of GBS. Adapted from (Doran and Nizet, 2004; Lindahl et al., 2005; Maisey et al., 2008a; Rajagopal, 2009; Vornhagen et al., 2017).

Virulence Factors	Mode of action	Genes
Pore-forming toxins		
CAMP Factor (Christie Atkins Munch Peterson)	. Binds to GPI anchored proteins, forms pores in host-cell membrane causing direct tissue injury; . Binds to IgC, IgM, decreasing antibody function;	*cfb*
β-Hemolysin/cytolysin + Hemolytic pigment (Ornithine rhamnolipid pigment)	. Induces inflammatory responses, namely sepsis syndrome, by cytokine release and apoptosis; . Impairs cardiac and liver function; . Promotes invasion of host cells, by the formation of pores cell membranes in and triggers host-cell lysis; . Antioxidant effect of the hemolytic pigment neutralizes hydrogen peroxide, superoxide, hypochlorite and singlet oxygen, and thereby provides a shield against several elements of phagocyte oxidative burst killing; (β-Hemolysin/cytolysin biosynthesis is associated with the production of this hemolytic orange pigment)	*cylE and cyl* operon
Factors for immune evasion		
Capsular polysaccharide	. Impairs complement C3 deposition and activation; . Prevents immune recognition through molecular mimicry of host-cell surface sialic acid epitopes, blocking opsonophagocytic clearance;	*cpsA-L* *neuA-D*
C5a peptidase	. Prevents neutrophil recruitment due to cleavage of complement C5a; . Promotes adherence by binding to extracellular matrix attachment fibronectin and epithelial cells;	*scpB*
Host-cell adherence & invasion		
Pili	. Promotes adherence by binding to host-cells; . Promotes resistance to antimicrobial peptides through an unknown mechanism;	*PI-1* *PI-2a* *PI-2b* PI: Pilus island
C protein (α and β)	. Human cervical epithelial cell adherence and invasion. It specifically interacts with host cell glycosaminoglycan (GAG) on the epithelial cell surface to promote bacterial internalization; . Blocks intracellular killing by neutrophils, gaining resistance to phagocytic clearance; . Non-immune binding of IgA	*bca* (α) *bac* (β)
Fibrinogen-binding proteins A and B	. Promotes adherence of GBS to host cells by binding to extracellular matrix fibrinogen;	*fbsA* *fbsB*
Alpha-like protein family	. Binds to epithelial cells; . Suffers antigenic variation as evasion mechanism of antibody detection;	*bca, eps, rib, alp2, alp3, alp4*
Hyaluronate lysase	. Cleaves hyaluronic acid, degrading the extracellular matrix and invading host cells, promoting the spreading of GBS. The degraded components of hyaluronic acid are immunosuppressive.	*hylB*

1.4.1. Capsular Polysaccharide (CPS)

The capsular polysaccharide (CPS) is a major virulence factor of GBS, with most isolates being encapsulated. The capsular phenotype (serotype) is frequently used to classify GBS and essential for epidemiological characterization.

The GBS CPS is a complex polysaccharide present on the exterior of the bacterial wall and its composition is a combination of four of five monosaccharides: glucose, galactose, N-acetylglucosamine, sialic acid and rhamnose (with the exception of serotype VI that has only three of these five sugars in its composition), which form different oligosaccharide repeating units (Paoletti et al., 2006).

So far, ten capsular types were identified, (Ia, Ib and II to IX), known to be structurally distinct and antigenically unique, all possessing a terminal sialic acid (Doran and Nizet, 2004; Paoletti et al., 2006). This terminal side-chain is a key contributor of CPS as a major virulence factor by means of immune evasion and interference with complement mediated killing (Brochet et al., 2006; Doran and Nizet, 2004; Rubens et al., 1987).

The evasion to the host's immune system results from the sialylation of the CPS, given that sialic acid is widely present on glycans, not only of human cells but also in most vertebrate cells, being a sugar epitope broadly displayed on the surface of all mammalian cells, for which reason it is a classic case of molecular mimicry, where the host does not recognize it as a foreign agent (Doran and Nizet, 2004; Rajagopal, 2009).

Regarding the interference with complement mediated killing, the sialylated CPS inhibits the alternative pathway-mediated opsonophagocytosis. The alternative complement pathway serves as a primary recognition mechanism in the nonimmune host for a variety of microbial polysaccharides (Edwards et al., 1982). Deposition of complement C3 on the bacterial surface, with subsequent cleavage and degradation to opsonically active fragment C3b, is pivotal to host defense against invasive bacterial infection (Doran and Nizet, 2004). Terminal sialic acid residues are nonactivating surfaces for the alternative pathway, and they modulate its activation, thereby blocking formation of the alternative pathway C3 convertase C3bBb (Edwards et al., 1982). Therefore sialic acid-rich CPS prevents complement factor C3 deposition, consequently inhibiting opsonophagocytosis of GBS (Rajagopal, 2009).

The capsular polysaccharide is encoded by the *cps* gene cluster, composed of 16–18 genes, which are responsible for the synthesis, cell wall attachment and regulation of the GBS CPS (Berti et al., 2014). The *cps* cluster possesses polysaccharide-specific genes encoding glycosyltransferases and polymerases, which are different depending on the serotype, and has an adjacent group of conserved genes (Cieslewicz et al., 2001). The overall operon structure is similar in all ten identified serotypes, with conserved genes flanking genes encoding enzymes unique to a specific capsular serotype (Cieslewicz et al., 2001; Creti et al., 2004).

Although the sialylated nature of the CPS is a mean to evade the host's innate immune system, the capsule is a target for specific antibodies that can defeat its antiphagocytic properties, conferring the host with protective immunity against GBS infection, so this serotype-specific immune pressure may have been the cause for the emergence of new capsular types (Cieslewicz et al., 2005). On the other hand, the similarity in polysaccharide structure strongly suggests that the evolutionary pressure toward

antigenic variation exerted by acquired immunity is counterbalanced by a survival advantage conferred by conserved structural motifs of the GBS polysaccharides (Cieslewicz et al., 2005).

1.4.2. Surface Proteins

Colonization by GBS depends on adherence to host cell surfaces, which is crucial for GBS pathogenesis, promoting subsequent host cell invasion. The combined diversity of surface and secreted proteins provide GBS with the opportunity to interact with different types of extracellular matrices (Brochet et al., 2006), being able to adhere and invade a number of host cells, including vaginal epithelial cells, lung epithelial and endothelial cells. These interactions often involve the initial binding of GBS to extracellular matrix proteins, which facilitate subsequent interactions with host-cell surface and entry into the host cell (Rajagopal, 2009).

1.4.2.1. C Protein (α and β components)

The C protein is a unique GBS surface protein antigen, that was first identified in 1969 by Wilkinson and Moody (Lindahl et al., 2005; Michel et al., 1991; Paoletti et al., 2006). The C protein is a protein complex that is constituted by two distinct components: the trypsin-resistant α protein and the trypsin-sensitive β protein usually designated by alpha C protein (ACP) and beta C protein (BCP), respectively (Lindahl et al., 2005). These proteins are encoded by different genes and are independently expressed, yet both have the ability to elicit protective immunity (Lindahl et al., 2005; Michel et al., 1991; Paoletti et al., 2006). While not all GBS isolates carry the C protein, the GBS isolates that do appear to be more resistant to intracellular killing by phagocytes (Doran and Nizet, 2004; Lindahl et al., 2005; Michel et al., 1991; Rajagopal, 2009).

The alpha C protein mediates GBS invasion of human cervical epithelial cells (Nizet and Rubens, 2006). ACP promotes GBS internalization into host-cells through its interaction with the host-cell glycosaminoglycan, and it can also promote GBS invasion of the host-cells by binding to α1β1-integrins on the epithelial cell surface (Baron et al., 2004; Maisey et al., 2008a; Rajagopal, 2009). The gene that encodes ACP is the *bca* gene, and its nucleotide sequence contains tandem repeating units, making up most of its DNA sequence (Michel et al., 1992; Nizet and Rubens, 2006). ACP is commonly found on GBS serotypes Ia, Ib and II and is infrequent in serotype III (Rajagopal, 2009).

The beta C protein is capable of binding the Fc portion of the human IgA, and IgA deposited nonspecifically on the bacterial surface probably inhibits interactions with complement, allowing GBS to evade human immune responses (Maisey et al., 2008a; Nizet and Rubens, 2006; Yang et al., 2007). BCP is found on almost all serotype Ib GBS isolates, as well as on some isolates of types Ia, II, and V, but almost never on serotype III (Yang et al., 2007).

1.4.2.2. Alpha-like Protein Family (Alp)

In the same genomic locus of the gene *bca*, another gene was detected, resulting in the identification of a new protein, the Rib protein. This protein had a similar structure and sequence to the ACP, creating a novel family of bacterial surface proteins, the alpha-like protein (Alp) family (Stalhammar-Carlemalm, 1993; Wästfelt et al., 1996). Currently, there are six members of the Alp family: the α, Rib, Eps (also known as Alp1), Alp2, Alp3 (also known as R28) and Alp4 (Creti et al., 2004). A GBS isolate expresses only one member of the surface protein gene family, since the different Alp proteins are encoded by mutually exclusive allelic genes (Kong et al., 2002; Lachenauer et al., 2000).

All members of the Alp family contain large internal tandem repeats, and these regions are highly conserved (Creti et al., 2004; Lindahl et al., 2005). The Alp proteins are therefore encoded by stable mosaic genes, generated by a recombination of modules at the same chromosomal locus. For all members of the Alp protein family, the proteins were shown to vary in size among different isolates, with the size of the protein being proportional to the number of repeats it contained (Lachenauer et al., 2000).

The number of tandem repeats of the surface protein of a GBS strain is important for pathogenesis (Lindahl et al., 2005). Deletions in the repetitive region of the Alp were evidenced in some GBS isolates evading the host's immune system by losing some of the repeating units that resulted in consequent loss of epitopes, leading to less antibody binding, and giving GBS the opportunity to escape antibody mediated host immunity (Gravekamp et al., 1996; Paoletti et al., 2006). Nevertheless, some isolates often contain multiple tandem repeats, suggesting that longer proteins enhance virulence in humans (Gravekamp et al., 1998, 1997; Lindahl et al., 2005). Therefore, the number of Alp tandem repeats in a GBS strain is an adaptive mechanism that is thought to be modulated according to the pretended approach in the pathogenic process.

1.4.3. Pili

Pilus-like structures have mostly been described and well-studied in Gram-negative pathogens, although these structures have also been identified in a few Gram-positive pathogens (Lauer, 2005; Rosini et al., 2006). Pili are long, filamentous, multimeric macromolecules found on the bacterial surface and their function is to facilitate adherence and attachment of the pathogen to host cells, required for efficient colonization (Dramsi et al., 2006; Rajagopal, 2009; Rosini et al., 2006). The comparison of GBS genomes revealed the existence of genetic islands comprising the necessary components for the formation of pilus-like structures and two genetic loci were identified in GBS, designated as pilus island 1 (PI-1) and pilus island 2 (PI-2). PI-2 exists in two variant forms known as PI-2a and PI-2b (Cozzi et al., 2015; Margarit et al., 2009; Rosini et al., 2006). All GBS isolates carried at least one or a combination of two pilus islands, where PI-2 is always present and PI-1 may or not be present (Cozzi et al., 2015).

The structure and composition of the pilus islands consists of genes encoding five proteins: three structural pilus proteins - the major pilus subunit (backbone protein) that forms the pilus shaft, and two ancillary proteins that appear to be located at the pilus tip (AP1) and at the base (AP2). These three proteins possess a conserved C-terminal amino acid motif LP(X)TG for sortase recognition and

subsequent cell wall anchoring. The other genes encode two sortase enzymes, which catalyze the covalent polymerization of the three protein components of the pili, and are essential for pilus assembly and covalent attachment to the peptidoglycan cell wall (Cozzi et al., 2015; Maisey et al., 2008b; Margarit et al., 2009; Rosini et al., 2006).

Several studies have shown that GBS pili hold a multitude of functions such as adherence, invasion of host-cells and translocation of epithelial cells (Konto-Ghiorghi et al., 2009; Maisey et al., 2008b; Margarit et al., 2009; Pezzicoli et al., 2008), biofilm formation (Konto-Ghiorghi et al., 2009; Mandlik et al., 2008; Rinaudo et al., 2010) and resistance to host phagocytes and cationic antimicrobial peptides (Maisey et al., 2008b). The pili are capable of eliciting protective immunity, confirming a key role in bacterial pathogenesis, therefore being promising vaccine candidates (Cozzi et al., 2015; Maisey et al., 2008b; Margarit et al., 2009).

1.5. Vaccines

IAP has significantly reduced the incidence of EOD, but the incidence of LOD has remained stable or increased. The development of a vaccine is currently considered the most promising method of preventing GBS infection in all age groups. The prospects of an effective vaccine to decrease the incidence of GBS infection in newborns would be based in the stimulation of the production of functionally active antibodies in pregnant women that could cross the placenta and provide protection against neonatal GBS infection (Nuccitelli et al., 2015).

In the 1930s, Rebecca Lancefield demonstrated evidence of the protective nature of CPS-specific antibodies, by using CPS-specific polyclonal rabbit serum, mice could be protected against GBS infections (Lancefield, 1938). So far, the CPS remains the best studied vaccine target, and until recently was the only one for which human vaccine trials have been undertaken (Heath, 2016). In 1976 Baker and Kasper showed that maternal CPS-specific antibodies, transferred from the mother to the newborn by transplacental transmission of immunoglobulin (IgG), were able to confer the newborns with protection against GBS infections (Baker and Edwards, 2003). These findings promoted the development of a vaccine against GBS using CPS as antigen, suggesting that maternal vaccination could be a suitable effective strategy to prevent GBS infection in newborns (Chen et al., 2013; Nuccitelli et al., 2015). Since the 1980s various forms of vaccines were developed, but plain CPS based vaccines demonstrated to be insufficiently immunogenic, leading to the development of GBS polysaccharide-protein conjugate vaccines and protein-based GBS vaccines (Heath, 2016).

The conjugate vaccines use the CPS as the primary target and enhance immunogenicity by covalent conjugation of a protein carrier, such as the tetanus toxoid or CRM197 (a non-toxic mutant of diphtheria toxin). Protein vaccines use universal GBS surface proteins as the vaccine target, having the potential to confer broad protection across serotypes (Kobayashi et al., 2016). GlaxoSmithKline (previously Novartis) has sponsored phase I and II trials of a trivalent (Ia, Ib, III) CPS-CRM197 GBS conjugate vaccine, and are currently pursuing pre-clinical studies of a pentavalent (Ia, Ib, II, III, V) CPS-CRM197 vaccine for immunization in pregnancy to prevent subsequent invasive GBS disease in newborns and young infants (Kobayashi et al., 2016). Pfizer is also in an early phase of development with a candidate CPS-CRM197 vaccine for the prevention of GBS invasive disease in infants, through maternal immunization during pregnancy, and this vaccine is being developed using the platform developed for other conjugate vaccines (e.g., 13-valent pneumococcal conjugate vaccine) (Kobayashi et al., 2016). MinervaX is investigating a protein-based vaccine candidate (GBS-NN), based on a fusion protein of the N-terminal domains of Alpha-like proteins which has completed phase Ia studies and a phase Ib study is underway. To date, the results of these vaccines have shown favorable safety and immunogenicity (Kobayashi et al., 2016).

1.6. Antimicrobial Resistance in GBS

1.6.1. β-Lactams

Penicillin and other β-lactam antibiotics act by inhibiting the synthesis of the peptidoglycan, through binding covalently to, and consequently inhibiting, bacterial proteins known as penicillin binding proteins (PBPs). This irreversible inhibition of the PBPs prevents the final crosslinking (transpeptidation) of the peptidoglycan layer, disrupting cell wall synthesis. GBS isolates, invasive or colonizing, are considered universally susceptible to penicillin, ampicillin and other β-lactams, with these antibiotics being the first choice for treatment of all GBS infections, in all age groups, and also for IAP. Nevertheless, in 2008 two studies have described reduced penicillin susceptibility in GBS isolates, recovered from invasive infections in USA and Japan. These isolates revealed mutations in a penicillin-binding protein (PBP-2x), that resulted in reduced susceptibility to penicillin and elevated minimum inhibitory concentrations (MIC) (Dahesh et al., 2008; Kimura et al., 2008). The clinical impact of these findings is yet to be stated but the possible emergence of penicillin resistance in GBS is concerning.

1.6.2. Macrolides and Lincosamides

Macrolides affect protein synthesis at the ribosomal level by binding to the 50S ribosomal subunit, thereby blocking the elongation process. Resistance to macrolides in GBS presents as resistance only to erythromycin or to both erythromycin and clindamycin, and occurs mainly by two mechanisms, expression of the gene *erm* (erythromycin ribosome methylase) or of the gene *mef* (macrolide efflux). The *erm* gene encodes a methylase which modifies the target site of the macrolide in the ribosome, altering the binding site, which in turn confers broad-spectrum resistance to macrolides, lincosamides and streptogramin B, also known as the MLS_B phenotype (Leclercq, 2002). The expression of MLS_B resistance may be constitutive ($cMLS_B$) or inducible ($iMLS_B$), in the first case, the methylase is always produced, and in the later, the methylase is only produced in the presence of an inducer antibiotic, namely clindamycin. The *mef* gene encodes a membrane-bound pump that is responsible for the active efflux of the antibiotic, conferring resistance only to macrolides, being known as the M phenotype (Leclercq, 2002).

In GBS, macrolide resistance is conferred mostly by *erm*(B) gene and *erm*(TR), a subset of *erm*(A) gene (Brzychczy-Włoch et al., 2010; Castor et al., 2008; de Azavedo et al., 2001; Figueira-Coelho et al., 2004; Seo et al., 2010). An association between the *erm*(B) gene and the $cMLS_B$ phenotype, and of the *erm*(TR) gene and the $iMLS_B$ phenotype has been shown (Castor et al., 2008; Sadowy et al., 2010). Antibiotic resistance varies worldwide, depending on the geographical location and the GBS population, notwithstanding, most studies have identified an association between serotype V and erythromycin resistance (Castor et al., 2008).

There is also an uncommon resistance phenotype to lincosamides and streptogramins A, known as the LS_A resistance phenotype, reported for the first time in GBS in New Zealand (Malbruny et al., 2004). In this case, the resistance to lincosamides and streptogramins A is conferred by the *lsa(C)* gene, which

is an efflux pump (Malbruny et al., 2011). In previous years, GBS isolates presenting resistance to clindamycin only were found in Taiwan, suggesting the possible presence of this phenotype in the country (Ko et al., 2001; Wu et al., 1997).

A GBS isolate resistant only to clindamycin was found in Canada, although in this case this resistance was conferred by the *lnu(B)* gene (de Azavedo et al., 2001). This gene was previously identified in *Enterococcus faecium* and is responsible for lincosamide nucleotidylation, inactivating the compound and consequently conferring resistance to this antimicrobial only (Bozdogan et al., 1999).

1.6.3. Tetracycline

Tetracycline acts by inhibiting protein synthesis, by binding to the 30S ribosomal subunit at a point that blocks the attachment of tRNA to the mRNA-ribosome complex, therefore, it is considered bacteriostatic rather than bactericidal. In GBS, resistance to tetracycline is present in most isolates (Cunha et al., 2014; Lopes et al., 2018; Metcalf et al., 2017), being considered almost ubiquitous, probably due to the large use of tetracycline in the past (Cunha et al., 2014). This resistance is conferred by *tet* genes, namely the most frequent, *tet*(M) and *tet*(O), which encode proteins which protect the ribosome from the action of the antibiotic, and the less common, *tet*(K) and *tet*(L) which encode a tetracycline efflux pump (Chopra and Roberts, 2001). While tetracycline is no longer used for clinical treatment of GBS infections, its surveillance is important since tetracycline resistance genes are often found on the same mobile element as erythromycin resistance genes, particularly, the gene *erm*(B) is frequently found linked with *tet*(M) (Culebras et al., 2002).

1.6.4. Quinolones

The target of the quinolones are DNA gyrase and topoisomerase IV, the enzymes responsible for nicking, supercoiling, and sealing bacterial DNA during replication and transcription. By inhibiting bacterial DNA synthesis, these agents are bactericidal. Resistance to this antibiotic happens through point mutations leading to target modification, however, a single target modifying mutation does not usually confer significant resistance, rather resistance is a cumulative process, with increasing numbers of mutations generally corresponding with higher MICs (Woodford, 2005). These mutations usually occur in specific areas, known as the quinolone resistance determining regions (QRDR), of the target genes *gyr*A and *gyr*B (for DNA gyrase), and *par*C and *par*E (for topoisomerase IV). Still, mutations that confer fluoroquinolone resistance arise mostly in *gyr*A and *par*C and are unusual in *gyr*B and *par*E (Kawamura et al., 2003; Woodford, 2005). The first GBS isolates resistant to quinolones emerged in Japan and these quinolone-resistant isolates had double point mutations within the QRDR of *gyr*A and *par*C (Kawamura et al., 2003). Later on, quinolone resistant GBS isolates were reported in other countries (Hays et al., 2016; Murayama et al., 2009; Wehbeh et al., 2005; Wu et al., 2017).

1.6.5. Chloramphenicol

Chloramphenicol acts by inhibiting protein synthesis through binding to the 50S ribosomal subunit, blocking its action and preventing process of peptide chain elongation, and so it is considered bacteriostatic. Resistance to this antibiotic is mostly due to the synthesis of the chloramphenicol acetyltransferase (cat enzyme), encoded by the *cat* gene, which inactivates the antibiotic by chemical conversion, making it no longer able to bind to the ribosomes. In GBS, *cat* genes chromosomally integrated have been detected, and this acquisition happened through transposons and plasmids that carried these genes, acquired through horizontal gene transfer (Trieu-Cuot et al., 1993).

1.6.6. Aminoglycosides

Aminoglycosides act by inhibiting protein synthesis through binding irreversibly to the ribosome affecting the elongation process, namely the proofreading process, disturbing the proper translation of the mRNA. Aminoglycoside resistance mechanisms consist in alteration of the ribosomal binding sites, reduced drug uptake, likely due to membrane impermeabilization, and expression of aminoglycoside-modifying enzymes, which modify the drug inactivating it (Mingeot-Leclercq et al., 1999). They are the main contributors to high-level aminoglycoside resistance (HLAR) (Mingeot-Leclercq et al., 1999; Vakulenko et al., 2003). The first published high-level aminoglycoside resistance in GBS isolates was in France, over a decade ago (Poyart et al., 2003). Recently it was found in Portugal in neonates (Martins et al., 2017) and adults (Lopes et al., 2018), and also in China (Campisi et al., 2016) and Canada (Teatero et al., 2016).

1.7. Characterization of GBS isolates

Typing methods, phenotypic or genotypic, are used to characterize isolates in order to study the propagation and population dynamics of a certain pathogen. These methods enable the recognition of particularly virulent isolates, the relationships between isolates, their transmission patterns and to identify and analyze trends and changing patterns for the control of GBS disease in the human population (Ranjbar et al., 2014; van Belkum et al., 2007).

1.7.1. Phenotypic Methods

Phenotypic methods are used to group and classify microorganisms according to their resemblance in observable characteristics (phenotypes), like colony morphology, color and other macroscopic features, which result from the expression of their genotypes (van Belkum et al., 2007). The majority of phenotypic methods rely on more than just visible traits, but also what characteristics emerge in certain conditions, like the ability of isolates to grow or not in the presence of specific substances. The most frequent conventional phenotypic methods are serotyping (based on different surface antigens) and antibiotic resistance typing (susceptibility to a selection of antimicrobials) (van Belkum et al., 2007). Phenotypes are generally quite susceptible to changes in environmental conditions, and for that reason these methods require strict standardization of experimental conditions, to ensure consistent and accurate results (van Belkum et al., 2007).

1.7.1.1. Serotyping

Serotyping is a method used to differentiate microorganisms from the same species, based on the reaction of specific antibodies with expressed surface antigens. Serotyping is the most used method of phenotypic assessment in GBS and essential for epidemiological characterization. Latex agglutination is now standard for serotyping GBS, and it is based on specific polyclonal antibodies for the ten CPS (Yao et al., 2013). Some isolates may have a low expression of CPS or not express it, in which case are considered non-typeable (NT) (Benson et al., 2002; Yao et al., 2013).

1.7.1.2. Antimicrobial Susceptibility Testing (AST)

Antimicrobial Susceptibility Testing (AST) is dependent on the diversity, stability and relative prevalence of the detectable acquired resistance mechanisms in any particular isolate (van Belkum et al., 2007). It is used for the correct management of infections in patients, since its results are commonly used to select the appropriate therapy.

In research, AST is usually performed by the Kirby-Bauer disk diffusion method susceptibility test, and the purpose of this test is to determine the susceptibility or resistance of pathogenic bacteria to

various selected antimicrobial compounds (Hudzicki, 2009). The pathogenic organism is grown on a plate of Mueller-Hinton agar, in which is placed various filter paper disks impregnated with the antimicrobial agent, usually at a standard concentration. After proper incubation under specific conditions, there may be a halo around the antimicrobial disk, designated inhibition diameter, which indicates the absence of growth. Measuring this diameter, and comparing it to reference tables, allows to determine if the microorganism is susceptible, intermediately susceptible or resistant to the antibiotic, as it is an indirect measure of the ability of that compound to inhibit that microorganism (Hudzicki, 2009). There is also another method used for antimicrobial susceptibility testing, the ETEST, which consists on the incubation of a microorganism with a predefined gradient of antibiotic concentrations on a plastic strip, originating an ellipse shaped zone of no growth, and where the ellipse meets the strip, can be read the MIC of the antibiotic, which is the lowest concentration of an antibiotic that prevents visible growth of bacterium. There can also be performed additional tests for the detection of resistance phenotypes such as the D-zone test, a disk approximation method which involves placement of an erythromycin and a clindamycin disks in close proximity on the surface of an agar plate. The reference tables with zone diameter and MIC interpretive criteria, the specific growth and incubation requirements and all the procedures are followed according to specific guidelines, the most well-known are the Clinical Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST). These standards are essential to ensure uniformity of the technique and the reproducibility of the results, and both the CLSI and EUCAST are responsible for updating and modifying the guidelines through a global consensus process, as pathogens develop new mechanisms of resistance and new antimicrobials are developed to fight these organisms (Hudzicki, 2009).

1.7.2. Genotypic Methods

Genotypic methods allow the identification of the bacterial isolates to the strain level, to determine the level of variation in their genomes and provide basic information about whole genome, precise nucleotide sequences (of one or more genes or intergenic regions), structure and overall composition. There is a wide variety of genotypic methods, like PCR-based typing methods, multilocus sequence typing (MLST) and whole genome sequencing (van Belkum et al., 2007).

1.7.2.1. PCR-Based Gene Profiling

Polymerase chain reaction (PCR) is a technique widely used for a large variety of typing methods with several specific applications in bacterial typing systems. It possesses an easily adjustable level of discrimination, being able to reach excellent discriminatory power and it is also highly reproducible, simple, not very costly with wide accessibility of equipment and reagents, producing results quickly.

Several PCR-based typing systems have been used to genotype GBS isolates, including molecular serotyping, surface protein gene profiling, detection of mobile genetic elements, and of antimicrobial resistance genes.

1.7.2.2. Multilocus Sequence Typing (MLST)

Multilocus sequence typing (MLST) is an unambiguous sequence-based typing method that facilitates the discrimination of microbial isolates by sequencing internal fragments (approximately 500 bp) of usually seven housekeeping genes and then comparing the obtained sequences with known alleles deposited at the MLST database (http://www.mlst.net and in the case of *S. agalactiae* https://pubmlst.org/sagalactiae) (Jones et al., 2003). An allele number is assigned to each sequence from each of the seven housekeeping genes, generating an allelic profile, taking into account that all unique sequences are assigned a new allele number in order of discovery (Urwin and Maiden, 2003). Each isolate is therefore characterized by an allelic profile, a seven-integer number, and each allelic profile is then assigned a sequence type (ST), and isolates with the same allelic profile are assigned to the same ST (Jones et al., 2003).

MLST is widely used because it provides a precise and unambiguous way of characterizing bacterial isolates and subsequently bacterial populations, it allows to determine levels of relatedness between isolates, by comparing allelic profiles and STs, and therefore the reconstruction of evolutionary events, being particularly suitable for epidemiological studies, as the data can easily be compared and accessed through the databases (Feil et al., 2004; Jones et al., 2003). All this is implemented through an algorithm, eBURST, that divides an MLST data set of any size into groups of related isolates and clonal complexes (CC), which are clusters of closely related allelic profiles, and predicts the founding allelic profile of each clonal complex through a set of rules (Feil et al., 2004).

The simplest model for the emergence of clonal complexes is that a certain clone increases in frequency in the population, by a fitness advantage or random genetic drift, becoming a founder clone. The descendants of the founder clone will initially remain unchanged in allelic profile, but over time variants will arise, by point mutation or recombination, in which one of the seven alleles has changed. 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, named double-locus variants (DLVs), and that differ at three of the loci, triple-locus variants (TLVs), and so on (Feil et al., 2004). Therefore, the presence of the founder clone in the population increases, existing a gradual diversification of its genotype, forming a cluster of phylogenetically closely related strains, resulting in a clonal complex.

eBURST can therefore be used to explore how bacterial clones diversify and can provide evidence concerning the emergence of clones of particular clinical relevance. Within each clonal complex, the rules identify which links between STs correspond to the most probable pattern of descent. However, the eBURST algorithm is not globally optimized, which can result in links within the clonal complexes, that violate the rules proposed, therefore goeBURST was designed to provide a global optimal solution, which corrects links that were not strictly following the eBURST rules (Francisco et al., 2009). A software implementation of the goeBURST algorithm is available at http://www.phyloviz.net/goeburst/ (Francisco et al., 2009).

1.7.2.3. Whole-Genome Sequence (WGS) Comparison

The breakthrough of DNA sequencing revolutionized several subjects in science and the concomitant development of technology, such as computational approaches, databases and computing power, leading to the emergence of new sequencing techniques that became more efficient and also less expensive. This phenomenon led to the sequencing of complete genomes, from pathogenic bacteria to complex eukaryotes. After the sequencing of several bacterial pathogens, the comparison of whole-genome sequences started, offered the possibility to assess genetic differences and resemblances within a bacterial species, providing insights on how genetic variability drives pathogenesis, namely the evolution of virulence mechanisms (Tettelin et al., 2005).

In 2002, the whole-genome sequence of GBS was revealed, in two different studies, evidencing a genome of approximately 2.2 Mbp long, encoding over 2100 genes (Glaser et al., 2002; Tettelin et al., 2002). Both studies presented similar results overall, and also both studies compared the GBS genome with the genomes of two related pathogens, *Streptococcus pyogenes* and *Streptococcus pneumoniae*, demonstrating a conserved backbone between these streptococci. Furthermore, it was observed a higher similarity between GBS and *S. pyogenes*, revealed by a high conservation of the chromosomal architecture. However, the GBS genome differed from the other streptococci in regions where genes were clustered, containing known and putative virulence genes, mostly encoding surface proteins, as well as genes related to mobile elements. These clusters showed characteristics of pathogenicity islands, which may have an important role in virulence acquisition, genetic diversity and adaptation to distinct niches in its human and animal hosts (Glaser et al., 2002; Tettelin et al., 2002). As some of these genes were associated with mobile elements, including bacteriophages, transposons, and insertion sequences, it supports the acquisition of virulence traits from other species (Tettelin et al., 2002).

Later, a study comparing multiple genomes from different GBS serotypes was published and in it the concept of “pan-genome” emerged (Tettelin et al., 2005). A bacterial species can be described by its ‘‘pan-genome’’, which includes a core genome containing genes present in all isolates, accounting for approximately 80% of any single genome, and a dispensable genome composed of genes absent from one or more isolates and genes that are unique to each strain (Tettelin et al., 2005). This genetic heterogeneity among GBS isolates, even of the same serotype, provided evidence that mechanisms of duplication, reassortment and acquisition have produced the genetic diversity within the species that has permitted GBS to express various combinations of virulence factors in order to adapt to new environmental niches and to emerge as a major human pathogen (Tettelin et al., 2002).

1.8. Molecular Epidemiology of GBS

1.8.1. Serotype Distribution

The capsular serotype is the classical method to classify GBS isolates and the monitoring of serotype distribution is essential for epidemiological and vaccine related studies (Kong et al., 2008).

It is known that serotypes Ia, Ib, II, III and V are the five-major disease-causing serotypes, responsible for 80 to 90% of all invasive GBS infections worldwide, however, it is possible to observe disparities in the geographical distribution of each serotype, as well as distinct serotype distributions according to the age group associated GBS infection (Kong et al., 2008; Le Doare and Heath, 2013; Rajagopal, 2009).

In a recent study data was collected from one hundred thirty-five scientific publications, reporting data from fifty-seven countries regarding the worldwide serotype distribution of GBS invasive infections in newborns. The results showed that the distribution of serotypes was consistent independently of the geographical location, where serotype III was the most frequent (61.5%), and 97% of all GBS invasive cases were caused by serotypes Ia, Ib, II, III, and V (Madrid et al., 2017). Figure 4 shows the percentage of GBS serotypes responsible for infections in newborns until three months of age, where serotype III is clearly predominant, followed by serotypes Ia, V, Ib and II (Madrid et al., 2017).

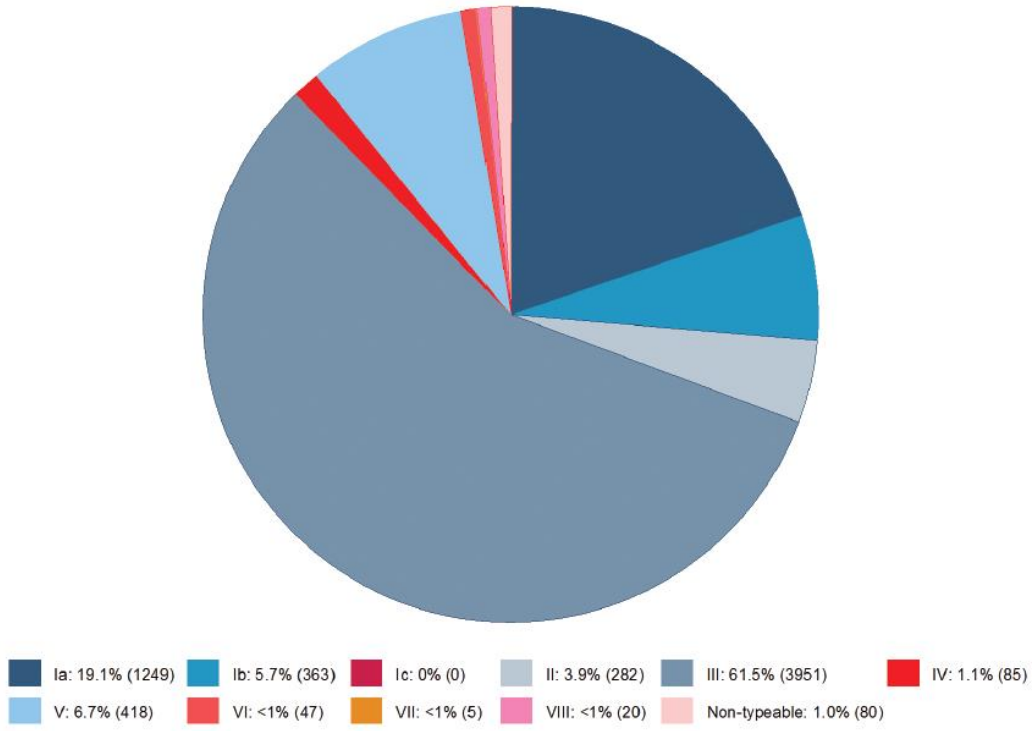

Figure 4 – Global distribution of GBS serotypes in invasive disease in newborns. Adapted from (Madrid et al., 2017).

There were some differences in the serotype distribution among EOD and LOD cases (Figure 5). In the same study, serotype III was the most frequent (47.4%) in EOD cases, followed by Ia, Ib, V and II. In LOD there was a considerable predominance of serotype III isolates (72.8%), although serotypes Ia, V, Ib and II were also found, being less frequent that in EOD cases (Madrid et al., 2017).

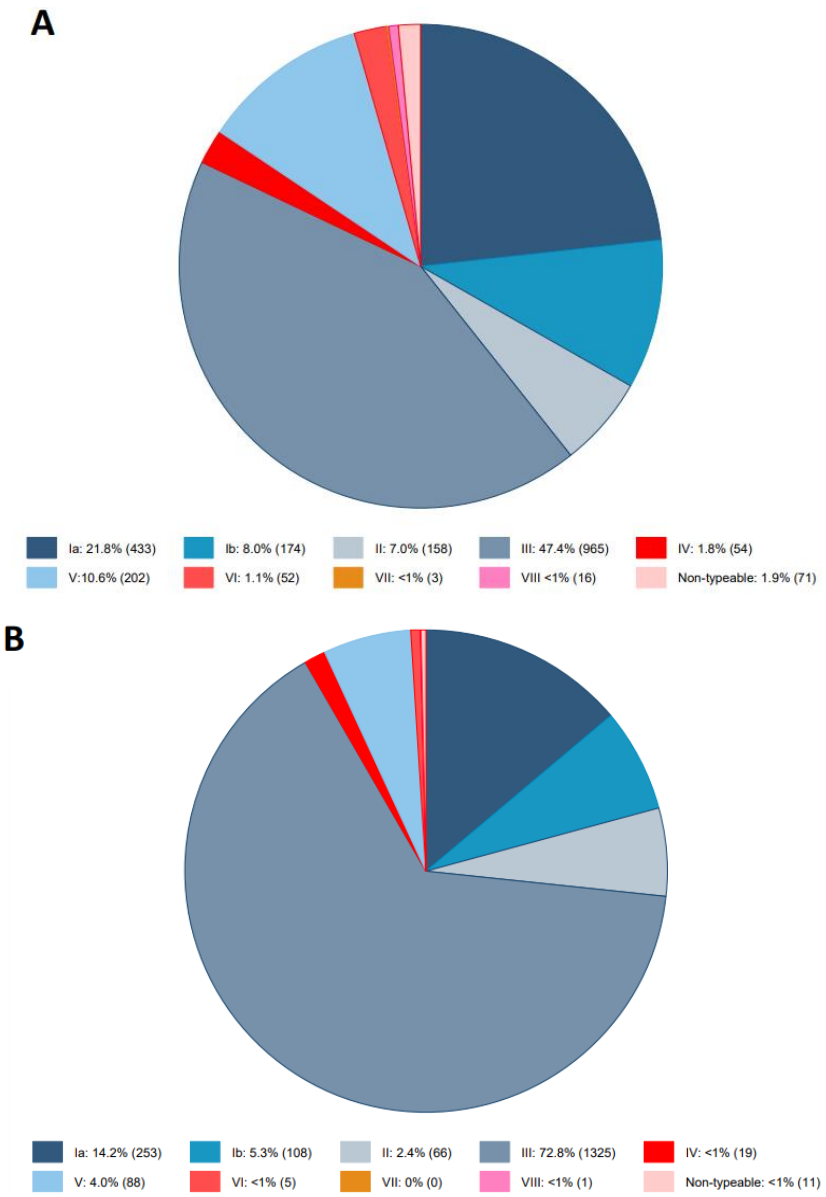

Figure 5 – Distribution of GBS serotypes for A) EOD and B) LOD GBS disease. Adapted from (Madrid et al., 2017).

Regarding pregnant women, serotype distribution in maternal GBS disease was expected to be similar to that of EOD, since GBS colonization is the main risk factor of EOD, due to GBS transmission to the newborn upon birth. However, while the most frequent serotypes were mostly the same, their relative proportions differed, with serotype Ia being the most prevalent (31%), followed by III, V, Ib, and II (Figure 6) (Hall et al., 2017).

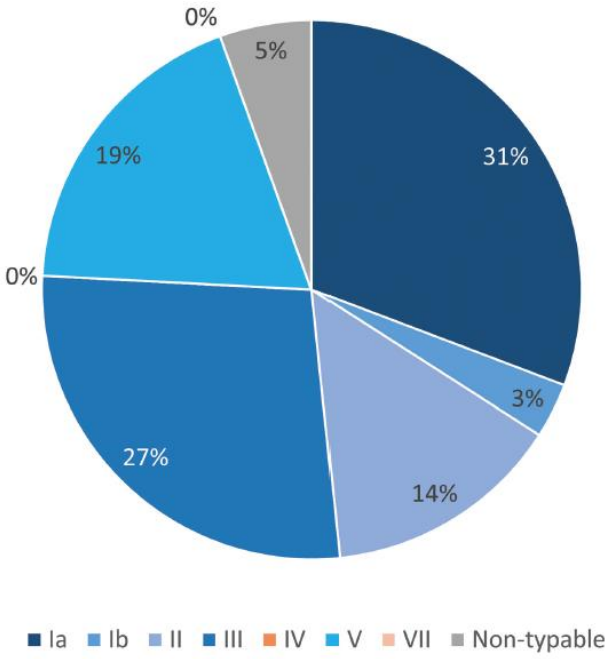

Figure 6 – GBS serotypes causing maternal GBS disease. Adapted from (Hall et al., 2017).

In contrast to newborn infections, where there has been a more conserved serotype distribution worldwide, with serotype III being the most frequent, in invasive disease in non-pregnant adults there is a significant difference between countries. In Japan, the most common capsular serotype was Ib (Morozumi et al., 2016). 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, 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 serotype Ia decreasing and serotype Ib increasing (Lopes et al., 2018). In Brazil, serotype Ia was also predominant (Dutra et al., 2014).

1.8.2. Genetic Lineages

The use of molecular typing methods, in particular MLST, has offered the possibility to infer phylogenetic relationships between strains and recognize genetic lineages. The combination of different typing methods helps to determine if different GBS genetic backgrounds have distinct virulence gene profiles that may be important for disease pathogenesis, such as tropism for a specific age group or host niche (Springman et al., 2009).

Particular associations between serotypes and surface proteins have been shown, particularly between Ia and *eps* and *bca*, Ib and *bca*, II and *bca* and *rib*, III and *rib* and V and *alp3* (Lindahl et al., 2005; Paoletti et al., 2006; Persson et al., 2008).

It has also been demonstrated a significant variation in the distribution of PI across phylogenetic lineages and populations, suggesting that pilus combinations impact host specificity and disease outcomes (Springman et al., 2014). Different pili combinations were found in different genetic lineages, the most prominent is an almost exclusive association between CC17 and PI-1+PI-2b in invasive neonatal disease (Martins et al., 2013).

A general correlation between CCs and serotypes is also evident: Ia with CC23, Ib with CC12, III with CC17 and CC19, V with CC1 and CC26 and the five most common CCs worldwide are CC1, CC10, CC17, CC19, CC23 (Brochet et al., 2006; Jones et al., 2003; Martins et al., 2013, 2012; Meehan et al., 2014; Springman et al., 2014).

The first recognized lineages worldwide were serotype III/ST17 and serotype III/ST19, the latter was mostly associated to colonizing isolates (Bisharat et al., 2005; Jones et al., 2003; Meehan et al., 2014), while serotype III/ST17 represents a successful clone with increased invasiveness in neonates, being acknowledged as the hypervirulent clone (Bisharat et al., 2005; Jones et al., 2003; Lin et al., 2006; Luan et al., 2005). Furthermore, serotype III isolates belonging to the hypervirulent CC17 have been significantly associated with meningitis, accounting for many EOD cases and even a higher number of LOD cases (Lazzarin et al., 2017; Manning et al., 2009; Meehan et al., 2014; Springman et al., 2009). The CC17 lineage is relatively homogeneous and therefore appears to have diverged independently with an exclusive set of virulence characteristics (Manning et al., 2009). Characterization of these lineages revealed the uniform presence of the *rib* gene in both, therefore known as serotype III/ST19/*rib* and serotype III/ST17/*rib* (Bergseng et al., 2009; Gherardi et al., 2007; Martins et al., 2017; Meehan et al., 2014; Sadowy et al., 2010). Additionally, the PI-1+PI-2b combination was highly related to CC17, and the PI-1+PI-2a combination was mostly present in CC19 (Campisi et al., 2016; Lazzarin et al., 2017; Martins et al., 2017, 2013; Springman et al., 2014).

Other worldwide recognized genetic lineages are serotype Ia/CC23/*eps*, and serotype V/CC1/*alp3*, to which most invasive disease cases among adults have been attributed, although CC23 has also been linked to neonatal invasive disease (Lamagni et al., 2013; Martins et al., 2017; Meehan et al., 2014; Teatero et al., 2014). In CC23 there is a predominance of serotype Ia/ST23/*eps*, mostly associated with PI-2a, although there is a sub-lineage constituted by a double-locus variant of ST23 represented by ST24/*bca*/PI-2a which has been mostly detected in the Mediterranean region (Gherardi et al., 2007; Martins et al., 2012, 2011). Within CC23, while ST23 and SLVs were dominant among EOD cases, ST24 and SLVs were associated with LOD, indicating that within the same CC, particular sub-lineages

may be better adapted to cause specific disease presentations (Martins et al., 2017, 2013; Meehan et al., 2014).

The serotype V/CC1/*alp3*/PI-1+PI-2a genetic lineage is usually dominant among serotype V isolates and has been mostly found among invasive disease cases in non-pregnant adults (Martins et al., 2017, 2012; Meehan et al., 2014).

The serotype Ib/CC12 genetic lineage although not abundantly has been present in most countries causing GBS disease in newborns, as well as in pregnant and non-pregnant adults (Hall et al., 2017; Lamagni et al., 2013; Madrid et al., 2017; Martins et al., 2017; Morozumi et al., 2016; Tazi et al., 2011). In Portugal, a new genetic lineage has recently emerged, in which serotype Ib was represented by CC1 and not the typical CC12 (Lopes et al., 2018). Some isolates with these characteristics where also recently found in Canada and USA, but with much less frequency (Metcalf et al., 2017; Neemuchwala et al., 2016). This genetic lineage is characterized by serotype Ib/CC1/*alp3*/PI-1+PI-2a, and in Portugal it has been mostly associated with GBS disease in adults, being also present in a few neonatal cases (Lopes et al., 2018; Martins et al., 2017).

The combined diversity of capsular polysaccharides, surface and secreted proteins identified in GBS impact its capacity to interact with different cell types. GBS has a wide repertoire of gene combinations that allow it to express various combinations of virulence factors, with the purpose of adapting to host immunity. Therefore molecular epidemiology is essential to understand the development and evolution of such characteristics and to increase awareness for proper management and treatment of GBS infections (Brochet et al., 2006).

2. 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.

3. Materials and Methods

3.1. 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.

3.2. 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.

3.3. 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.

3.4. 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 MLSB 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.

3.5. DNA Extraction

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

3.6. 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).

3.7. 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).

3.8. 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.

3.9. 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.).

4. Results

4.1. 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.

4.2. Capsular Serotypes

Serotyping results are presented in Table 2. 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.

Table 2 – Serotype distribution of the 446 GBS SSTI isolates

Serotypes	Elderly	Young	Total
Ia	63	79	142 (31.8 %)
Ib	26	44	70 (15.7 %)
II	13	21	34 (7.6 %)
III	21	34	55 (12.3 %)
IV	5	1	6 (1.3 %)
V	51	64	115 (25.8 %)
VI	1	1	2 (0.4 %)
IX	6	0	6 (1.3 %)
NT	9	7	16 (3.6 %)
Total Geral	**195**	**251**	**446**

NT – non-typeable

Regarding the serotype distribution, significant changes were observed throughout the study period (Figure 7). 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).

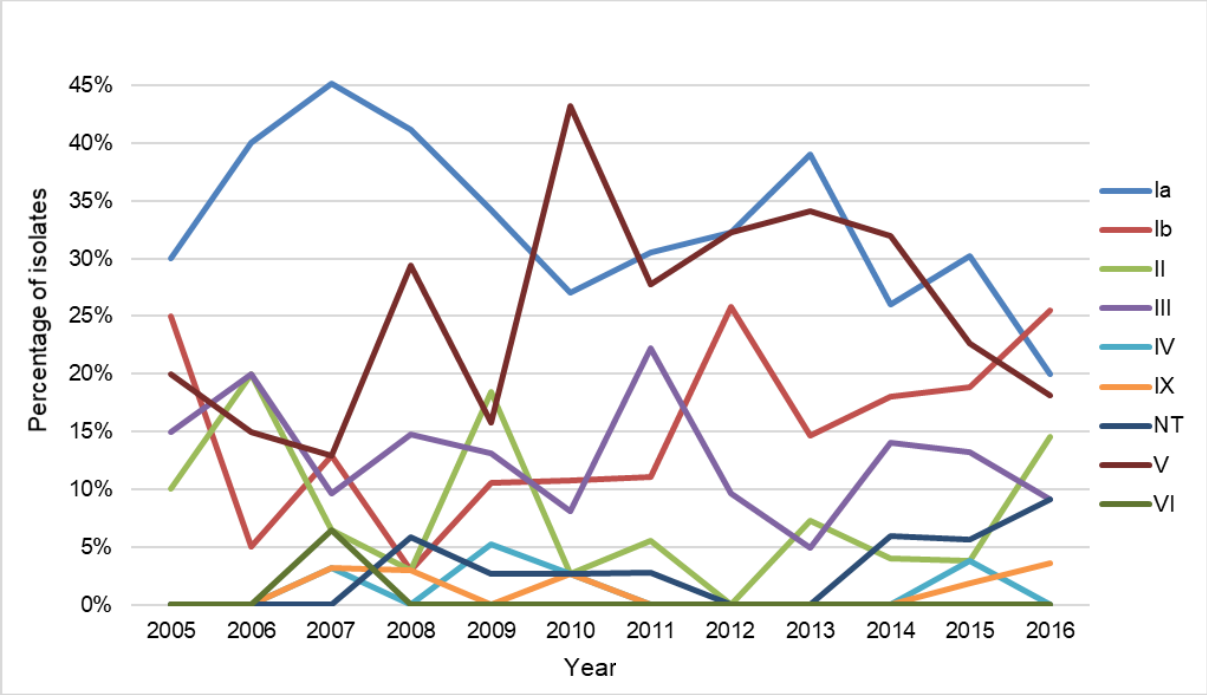

Figure 7 – Serotype distribution of the SSTI GBS isolates from 2005 to 2016.

4.3. Surface Protein Genes and Pilus Islands

The *alp3, bca, eps* and *rib* genes were equally frequent in the study collection (23-24%). The *alp2* gene was only present in 2% (n = 9) of the GBS isolates and in 3.6% (n = 16) of the GBS isolates no gene was detected. The *alp4* gene was not found in any GBS isolate.

The surface protein genes were differently distributed among serotypes (Figure 8). Serotype Ia was mostly associated with the *eps* and *bca* genes and serotype Ib with the *alp3* and *bca* genes. The *rib* gene was dominant among serotypes II and III. The *alp3* gene was the most frequent among serotype V isolates, but a small group of serotype V isolates did not carry any of the Alp genes tested.

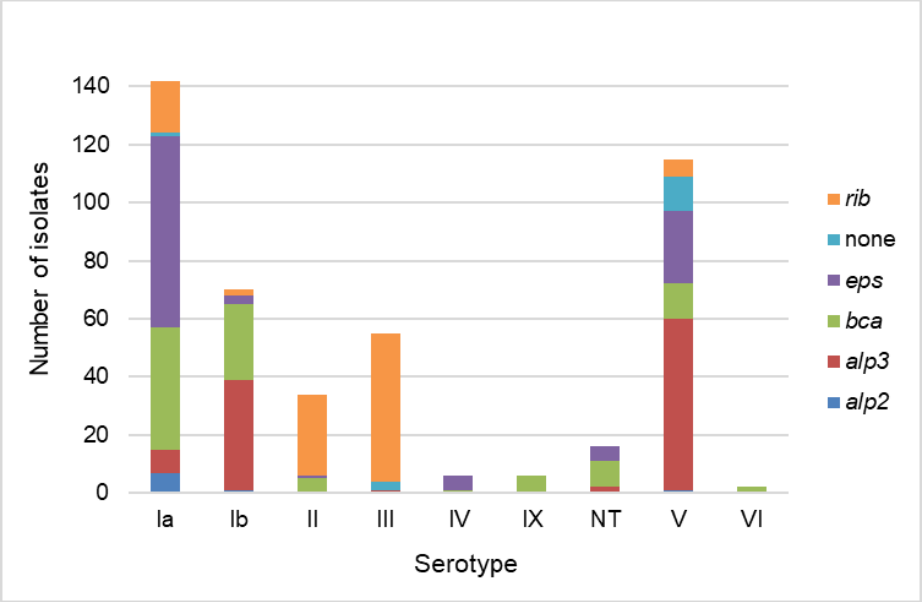

Figure 8 – Surface protein gene distribution by serotype of the SSTI GBS isolates.

Regarding the pilus islands distribution, the combination of PI-1+PI-2a was the most common, present in 60% of GBS isolates (n = 267), followed by the PI-2a only in 34% (n = 152), PI-1+PI-2b in 6% (n = 26) of the isolates, and 1 isolate carried the PI-2b only.

The pilus islands were also differently distributed among serotypes (Figure 9). Serotype Ia was mostly associated with PI-2a, as well as serotype IX. The PI-1+PI-2b was almost exclusively present in serotype III. The PI-1+PI-2a was dominant among serotype Ib, II and V isolates.

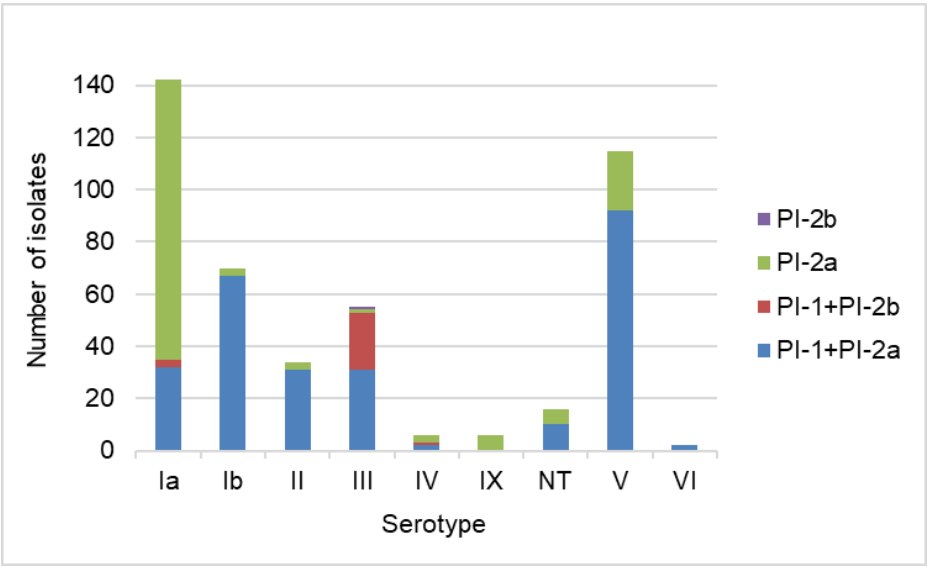

Figure 9 – Pilus islands distribution by serotype of the SSTI GBS isolates.

4.4. 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, $CI_{95\%}$ 0.749-0.790). Table 3 depicts the distribution of STs, serotypes, surface protein, pilus islands and antimicrobial resistance phenotypes and genotypes within the CCs. While a significant diversity of STs and serotypes was found, a small number of genetic lineages were the major contributors to SSTI.

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 10). 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.

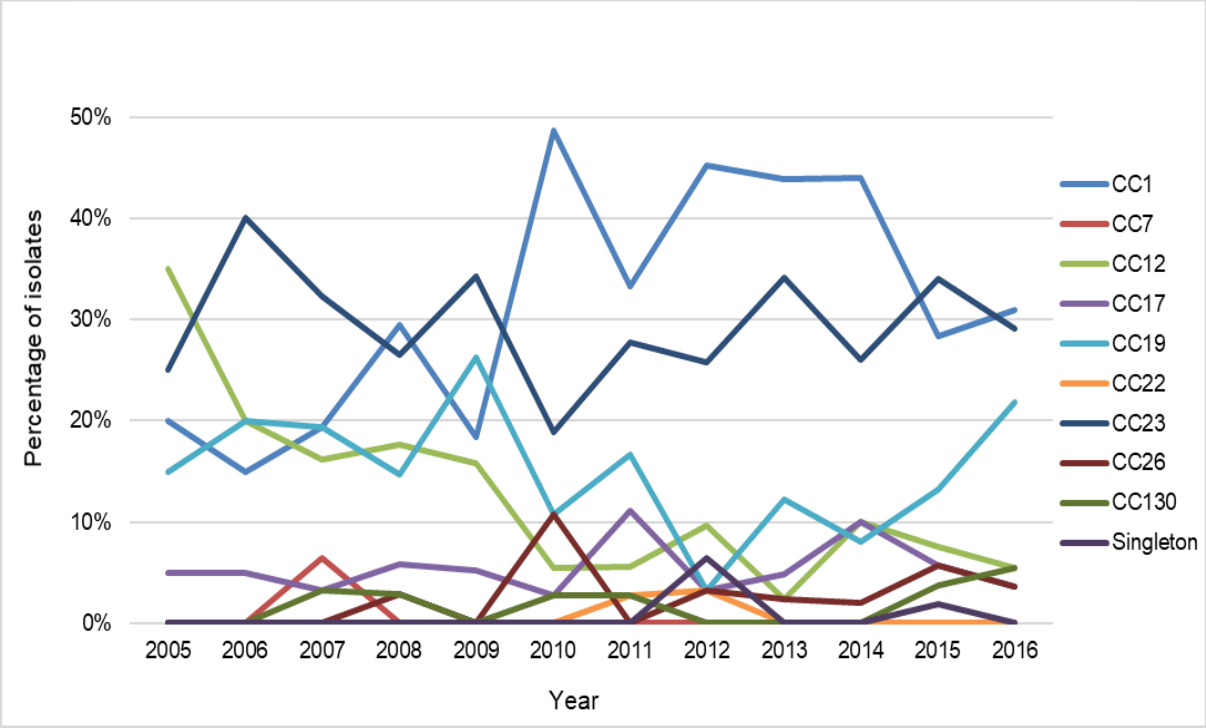

Figure 10 – CC distribution of the SSTI GBS isolates from 2005 to 2016.

Table 3 – Sequence type, serotype, surface protein, pilus islands, phenotype, genotype distribution of GBS SSTI isolates by clonal complex, from 2005 to 2016 (n = 446).

Clonal Complex (n)	Sequence Type (n)	Serotype (n)	Surface Protein gene (n)	Pili (n)	Resistance Phenotype (n)	Resistance Genotype (n)
1 (146)	1 (106)	Ia (7) Ib (38) III (1) V (56) VI (2) NT (2)	*alp3* (103) *rib* (1) *bca* (2)	PI-1+PI-2a (106)	$cMLS_B$ (54) $iMLS_B$ (10)	*erm*(TR) (11) *erm*(B) (53) *tet*(M) (60) S-ND (1)
	2 (29)	Ia (2) Ib (1) V (24) NT (2)	*eps* (28) *rib* (1)	PI-1+PI-2a (26) PI-2a (2)	$cMLS_B$ (1)	*erm*(B) (1) *tet*(M) (5)
	136 (1)	Ib (1)	*eps* (1)	PI-1+PI-2a (1)	$cMLS_B$ (1)	*erm*(TR) (1) *tet*(M) (1)
	196 (4)	Ib (1) IV (2) NT (1)	*eps* (4)	PI-1+PI-2a (4)	$cMLS_B$ (2)	*erm*(B) (1) *erm*(TR) (1) *tet*(M) (1) *tet*-ND (1) *aph(3')-III+ant(6)-Ia* (1)
	499 (1)	IV (1)	*eps* (1)	PI-1+PI-2b (1)	-	*tet*(M) (1)
	780 (1)	V (1)	*alp3* (1)	PI-1+PI-2a (1)	-	*tet*(M) (1)
	812 (1)	Ia (1)	*alp3* (1)	PI-1+PI-2a (1)	$cMLS_B$ (1)	*erm*(TR) (1) *tet*(M) (1)
	813 (1)	V (1)	*alp3* (1)	PI-1+PI-2a (1)	-	*tet*(M) (1)
	814 (1)	V (1)	*alp3* (1)	PI-1+PI-2a (1)	$cMLS_B$ (1)	*erm*(B) (1) *tet*(M) (1)
	1201 (1)	V (1)	*alp3* (1)	PI-1+PI-2a (1)	-	*tet*(M) *(1)*
7 (2)	7 (1)	Ia (1)	*bca* (1)	PI-1+PI-2b (1)	-	-
	808 (1)	Ia (1)	*bca* (1)	PI-1+PI-2b (1)	M (1)	*mef*(E) (1)
12 (48)	* (1)	Ia (1)	*bca* (1)	PI-1+PI-2a (1)	-	-
	8 (18)	Ib (15) V (2) NT (1)	*bca* (18)	PI-1+PI-2a (18)	$cMLS_B$ (1) M (2)	*erm*(B) (1) *mef*(E) (1) *tet*(M) (14) *tet*(M)+*tet*(O) (1)
	10 (21)	Ia (8) Ib (4) II (4) V (1) NT (4)	*bca* (18) *eps* (3)	PI-1+PI-2a (20) PI-2a (1)	$cMLS_B$ (1)	*erm*(B) (1) *tet*(M) (7) *aph(3')-III+ant(6)-Ia* (1)
	12 (5)	Ib (3) II (2)	*bca* (5)	PI-1+PI-2a (4) PI-2a (1)	$cMLS_B$ (1)	*erm*(B) (1) *tet*(O) (3) *tet*(M) (1) *aph(3')-III+ant(6)-Ia* (1)
	358 (1)	Ib (1)	*bca* (1)	PI-1+PI-2a (1)	$cMLS_B$ (1)	*erm*(TR) (1) *tet*(M) (1)
	770 (1)	Ib (1)	*bca* (1)	PI-1+PI-2a (1)	-	-
	1200 (1)	Ib (1)	*bca* (1)	PI-1+PI-2a (1)	-	*tet*(M) (1)
17 (25)	17 (21)	Ia (1) III (20)	*rib* (21)	PI-1+PI-2b (20) PI-2b (1)	$cMLS_B$ (2)	*erm*(B) (1) *erm*(B)+*mef*(E) (1) *tet*(M) (19) *tet*(M)+*tet*(O) (1) *aph(3')-III+ant(6)-Ia* (1)
	109 (1)	III (1)	*rib* (1)	PI-1+PI-2b (1)	-	*tet*(M) (1)
	287 (1)	III (1)	*rib* (1)	PI-1+PI-2b (1)	M (1)	*mef*(E) (1) *tet*(M) (1)
	291 (1)	III (1)	*rib* (1)	PI-1+PI-2b (1)	-	*tet*(M) (1)
	347 (1)	II (1)	*rib* (1)	PI-1+PI-2a (1)	-	*tet*(M) (1)
19 (67)	19 (25)	Ia (2) Ib (1) III (20) V (2)	*rib* (21) none (2) *eps* (2)	PI-1+PI-2a (25)	$cMLS_B$ (2) $iMLS_B$ (11)	*erm*(B) (2) *erm*(TR) (10) *mef*(E) (1) *tet*(M) (20) Lev-R (1) C-R (1) Lev-R+C-R (1)
	27 (2)	Ia (1) III (1)	*rib* (2)	PI-1+PI-2a (2)	-	*tet*(O) (1) *tet*(M)+*tet*(O) (1)
	28 (22)	Ia (1) II (20) III (1)	*rib* (22)	PI-1+PI-2a (20) PI-2a (2)	$cMLS_B$ (1) M (1) LS_A (1)	*erm*(B) (1) *mef*(E) (1) *lsa*(C) (1) *tet*(M) (18) *tet*(O) (1) *tet*(M)+*tet*(O) (1)

	110 (4)	Ia (1) V (3)	*rib* (4)	PI-1+PI-2a (2) PI-2a (2)	$cMLS_B$ (2)	*erm*(B) (2) *tet*(M) (2) *tet*(O) (1) *tet*(M)+*tet*(O) (1)
	267 (1)	II (1)	*rib* (1)	PI-1+PI-2a (1)	-	-
	286 (1)	III (1)	*rib* (1)	PI-1+PI-2a (1)	-	-
	335 (2)	III (2)	*rib* (2)	PI-1+PI-2a (2)	$iMLS_B$ (2)	*erm*(TR) (2) *tet*(M) (2)
	347 (5)	II (5)	*rib* (5)	PI-1+PI-2a (5)	LS_A (1)	*lsa*(C) (1) *tet*(M) (5)
	472 (1)	III (1)	*rib* (1)	PI-1+PI-2a (1)	-	*tet*(M) (1)
	807 (1)	III (1)	*rib* (1)	PI-1+PI-2a (1)	$cMLS_B$ (1)	*erm*(B) (1) *aph(3')-III* (1)
	809 (1)	III (1)	*rib* (1)	PI-1+PI-2a (1)	-	*tet*(M) (1)
	1202 (1)	II (1)	*rib* (1)	PI-1+PI-2a (1)	-	*tet*(M) (1)
	1205 (1)	V (1)	*rib* (1)	PI-1+PI-2a (1)	-	*tet*(M) (1)
22 (2)	22 (2)	V (2)	*bca* (2)	PI-2a (2)	$cMLS_B$ (1)	*erm*(B) (1) *tet*(M) (2)
23 (131)	23 (70)	Ia (63) Ib (1) III (2) IV (2) NT (2)	*eps* (57) *alp2* (7) *rib* (1) none (1) *eps* (4)	PI-2a (61) PI-1+PI-2a (9)	M (5)	*mef*(E) (5) *tet*(M) (58) *tet*(O) (1) *tet*-ND (1) Lev-R (1)
	24 (25)	Ia (21) IV (1) V (2) NT (1)	*bca* (24) *rib* (1)	PI-2a (20) PI-2a (4)	$cMLS_B$ (1)	*erm*(TR) (1) *tet*(M) (23) Lev-R (1)
	88 (1)	V (1)	*alp2* (1)	PI-1+PI-2a (1)	$cMLS_B$ (1)	*erm*(B) (1) *tet*(O) (1)
	144 (11)	Ia (11)	*rib* (11)	PI-2a (11)	-	*tet*(M) (9)
	262 (1)	Ia (1)	*eps* (1)	PI-2a (1)	-	*tet*(M) (1)
	498 (16)	Ia (12) V (4)	*bca* (16)	PI-2a (16)	$cMLS_B$ (1) M (2)	*erm*(B) (1) *mef*(E) (2) *tet*(M) (16)
	640 (2)	Ia (2)	*eps* (2)	PI-2a (2)	-	*tet*(M) (2)
	707 (1)	Ia (1)	*bca* (1)	PI-2a (1)	-	*tet*(M) (1)
	771 (1)	Ia (1)	*alp2* (1)	PI-1+PI-2a (1)	-	-
	1199 (1)	NT (1)	*bca* (1)	PI-2a (1)	-	*tet*(M) (1)
	1203 (1)	Ia (1)	*eps* (1)	PI-2a (1)	-	-
	1204 (1)	NT (1)	*bca* (1)	PI-2a (1)	-	*tet*(M) (1)
26 (13)	26 (12)	V (12)	none (12)	PI-2a (12)	$iMLS_B$ (2)	*erm*(TR) (2) *tet*(M) (8) *tet*(M)+*tet*(L) (1)
	811 (1)	Ia (1)	none (1)	PI-2a (1)	-	*tet*(M) (1)
130 (9)	130 (9)	Ib (1) V (1) IX (6) NT (1)	*bca* (9)	PI-2a (9)	-	*tet*(M) (1)
Singleton (3)	314 (1)	Ia (1)	*eps* (1)	PI-1+PI-2a (1)	-	*tet*(M) (1)
	529 (2)	Ib (1) III (1)	*rib* (2)	PI-1+PI-2a (2)	-	*tet*(M) (1) *tet*(O) (1)

*: GBS isolate with a deletion of 408bp in the *atr* allele; NT: non-typeable; PI: pilus island; MLS_B: macrolides, lincosamides and streptogramins B resistance, which can be expressed constitutively ($cMLS_B$) or inducibly ($iMLS_B$); M: resistance to macrolides; LS_A: resistance to lincosamides; S-ND: streptomycin not determined (phenotypically resistant to streptomycin but did not carry any of the resistance genes tested); C-R: resistance to chloramphenicol; Lev-R: resistance to levofloxacin; *tet*-ND: tetracycline not determined (phenotypically resistant to tetracycline but did not carry any of the resistance genes tested).

4.5. 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 11). 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 (Table 3). 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 (Table 3). 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 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 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.

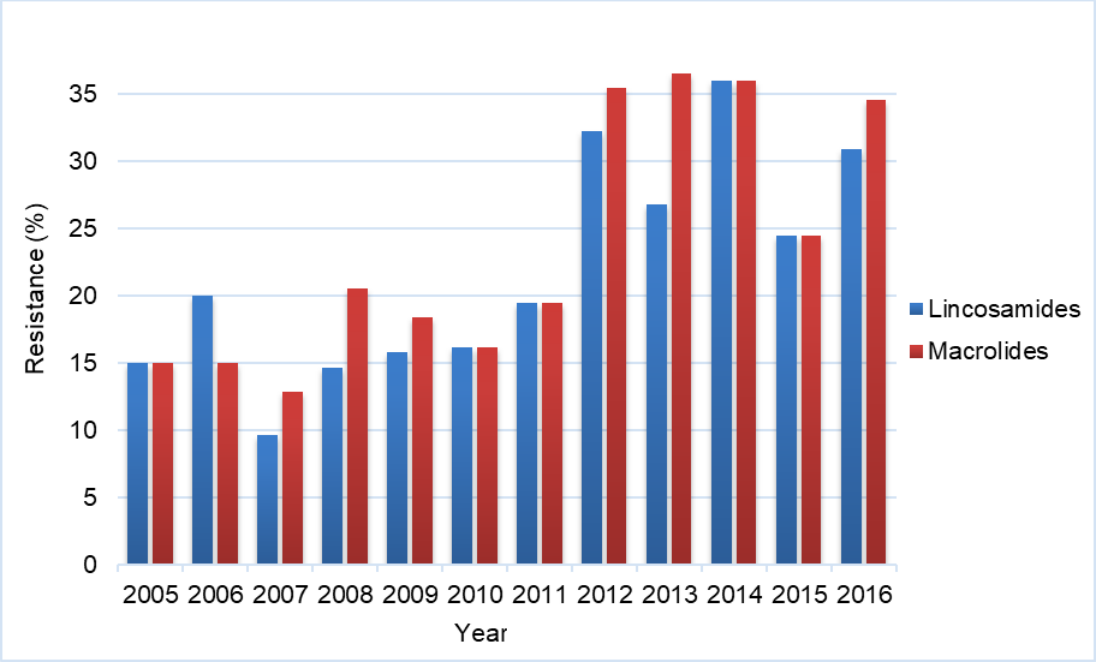

Figure 11 – Macrolide and lincosamide resistance rates of GBS SSTI isolates, from 2005 to 2016.

5. 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, 2001b; 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 from 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 results showed that serotypes Ia, V, Ib, III, and II were responsible for over 85% of SSTI in Portugal (Table 2) 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 7), 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 and Spain (Sadowy et al., 2010, 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 as 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 all 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 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.

6. Conclusion

The pivotal observation in this work was the change in serotype distribution throughout the study period, where there was a decrease of the dominant serotype Ia and an increase of serotype Ib. The serotype Ib/CC1 genetic lineage is established in Portugal, and in recent years it has become the dominant lineage, responsible not only for SSTI, but also invasive disease in non-pregnant adults (Lopes et al., 2018). Furthermore, the fact that this lineage is highly resistant to macrolides and lincosamides emphasizes the need for serotyping and antimicrobial susceptibility testing as standard procedure for proper antimicrobial therapy. The development of a vaccine is already under way and it would be the best approach to prevent GBS disease in both adults and newborns.

The reason why this genetic lineage has become dominant in Portugal while it does not appear to be particularly predominant elsewhere is still not clear. The identification of some GBS isolates belonging to this genetic lineage in Japan (Morozumi et al., 2016), Canada (Neemuchwala et al., 2016) and the USA (Metcalf et al., 2017) suggests that the capsular switching event responsible for this genetic lineage may have taken place in other locations. The increase of frequency of this genetic lineage in other regions and countries may lead to an overall increase in macrolide and lincosamide resistance.

This study highlights the importance of epidemiological surveillance and the continuous need for epidemiology studies. Epidemiological surveillance enables the monitorization of serotype/genotype combinations, already known or novel, and of antimicrobial resistance, leading to the proper management and treatment of GBS infections.

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