Group B *Streptococcus* as an invasive pathogenic in pregnant women

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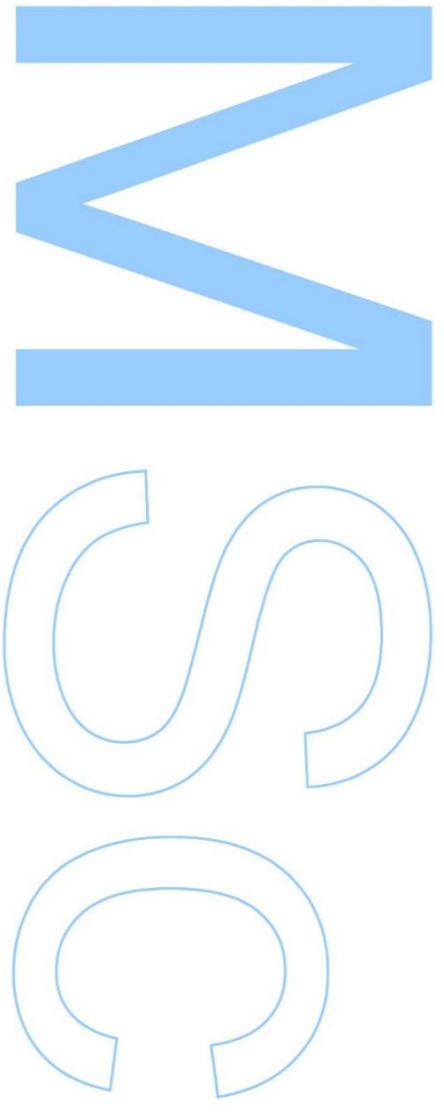

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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Resumo

Streptococcus agalactiae comumente denominado por *Streptococcus* do Grupo B (GBS) é responsável por infeções invasivas de recém-nascidos com alta morbilidade e mortalidade. As diretrizes de 2010 do Controlo e Prevenção de Doenças recomendam o rastreio de GBS em todas as mulheres grávidas entre as 35 e 37 semanas de gestação como prevenção da doença perinatal. A profilaxia antibiótica intraparto (IAP) pode diminuir significativamente as doenças neonatais por GBS; portanto, é importante identificar a sua presença. No entanto, continua a ser um relevante problema de saúde pública, causando abortos espontâneos e nados mortos. As vacinas serótipo-específicas têm o potencial de erradicar a doença e prevenir a infeção. À medida que são desenvolvidas, é importante identificar as linhagens genéticas responsáveis pelas infeções invasivas de GBS.

Os objetivos do trabalho apresentado nesta tese foram (1) rever o impacto europeu do GBS, (2) avaliar as ocorrências de GBS num hospital local e (3) avaliar a diversidade genética de isolados de GBS recuperados da colonização vaginal em mulheres grávidas entre as 35 e 37 semanas de gestação.

Para atingir estes objetivos foi realizado um estudo de revisão sobre o impacto do GBS a nível Europeu. Esta revisão inclui sessenta e cinco estudos que representam um total de 20 países Europeus. Taxas de 6,5 % a 36 % de portadores de GBS foram encontradas nas populações estudadas. O serótipo III (37 %) foi o serótipo mais frequente entre os países estudados, seguido do serótipo Ia (18 %). O nível de resistência de *Streptococcus agalactiae* à eritromicina e à clindamicina, dois antibióticos alternativos utilizados em casos de alergia à penicilina, tem crescido progressivamente.

Além disso, dados entre 2011 e 2016, fornecidos pelo Hospital Pedro Hispano (HPH), foram analisados para avaliar a ocorrência, ao longo do tempo, de GBS em mulheres grávidas entre as 35 e 37 semanas de gestação e em neonatos hospitalizados de mulheres grávidas entre as 21-41 semanas de gestação. Um total de 1377 (21 %) grávidas entre as 35-37 semanas de gestação mostraram ser positivas para o GBS. A incidência de sepsis neonatal foi de 8,7 (95 % IC: 7,0-10,8) por 1000 nados vivos (LB). Foi também realizado um estudo laboratorial em que 67 isolados de GBS diferentes foram caracterizados serologicamente, de forma a determinar os serótipos mais comuns. O serótipo III (22,4 %) foi o mais frequente seguido pelo serótipo Ia (19,4 %) e serótipos Ib e V (ambos com 17,9 %).

No geral, os resultados apresentados nesta tese sugerem que o GBS continua a ser um importante problema de saúde pública com altas taxas de portadores apresentadas no HPH e em alguns países europeus.

Serão necessários mais estudos epidemiológicos para compreender a evolução das estirpes de *S. agalactiae*, permitindo a formulação de uma vacina efetiva para a prevenção de infeções GBS.

Palavras-chave

Streptococcus do Grupo B, colonização bacteriana, mulheres grávidas, septicemia neonatal, profilaxia antibiótica de intraparto, prevenção.

Abstract

Streptococcus agalactiae commonly designated Group B *Streptococcus* (GBS) is responsible for invasive infections of newborns with high morbidity and mortality. The 2010 Centers for Disease Control and Prevention guidelines recommend the screening of GBS in all pregnant women between 35 and 37 weeks of gestation as a prevention of perinatal GBS disease. Intrapartum antibiotic prophylaxis (IAP) can significantly decrease neonatal GBS diseases; therefore, it is important to identify GBS presence. However, it remains an important public health problem causing spontaneous abortions and stillbirths as well. Serotype-specific vaccines have the potential to eradicate the disease and prevent infection. As these are being developed it is important to identify the genetic lineages responsible for GBS invasive infections.

The aims of the work presented in this thesis were (1) to review the European GBS impact, (2) to evaluate the occurrences of GBS in a local hospital and (3) to assess the genetic diversity of GBS isolates recovered from vaginal colonization in pregnant women between 35 and 37 weeks of gestation.

To achieve these objectives, a state of the art revision study on the impact of GBS at European level was carried out. This review includes sixty-five studies representing a total of 20 European countries. Rates from 6.5 % to 36 % of GBS carriers were found in the study populations. Serotype III (37 %) was the most frequent serotype among the studied countries followed by serotype Ia (18 %). GBS resistance to erythromycin and clindamycin has been increasing, the two antibiotic alternatives to penicillin used in cases of allergy.

Furthermore, data from 2011 to 2016 provided by Hospital Pedro Hispano (HPH) were analyzed to assess the occurrence over time of GBS in pregnant women between 35 and 37 weeks of gestation and in hospitalized neonates from pregnant women between 21-41 weeks of gestation. A total of 1377 (21 %) screened pregnant women between 35-37 weeks of gestation were found to be GBS positive. The incidence of neonatal sepsis was 8.7 (95 % CI: 7.0–10.8) cases per 1000 live births (LB). In addition, a laboratorial study on the serotype characterization of 67 different GBS isolates was performed, in order to determine the most common serotypes. Serotype III (22.4 %) was the most frequent followed by serotype Ia (19.4 %) and serotypes Ib and V (both with 17.9 %).

Globally, the results presented in this thesis suggest that GBS continues to be an important public health problem with high GBS carriers rates presented in HPH and in some European countries.

Further epidemiological studies will be necessary to understand the evolution of *S. agalactiae* strains, allowing the design of an effective vaccine for prevention of GBS infections.

Keywords

Group B *Streptococcus*, bacterial colonization, pregnant women, neonatal sepsis, intrapartum antibiotic prophylaxis, prevention.

Thesis outline

The main purpose of the work presented in this thesis was to understand the behavior of *Streptococcus agalactiae* with particular focus on its epidemiology, microbiology and prevention.

Chapter 1 introduces the thematics with a briefly revision of essential aspects about *S. agalactiae* disease.

Chapter 2 provides the objectives and a workplan that resumes the methodology used to obtain the results presented in Chapter 3 and 4.

Chapter 3 provides a literature revision about the thematic focusing on (1) procedures/ methodologies used (screening, culturing and antibiotic prophylaxis), (2) maternal GBS colonization, (3) serotype distribution and (4) antimicrobial resistances at European level. This chapter is under submission for publication as a review paper.

Chapter 4 presents a temporal analysis (between 2011 and 2016) on the number of cases of GBS registered in the hospital HPH from:

i. Pregnant women between 35 and 37 weeks of gestation
ii. Hospitalized neonates from pregnant women between 24-41 weeks of gestation.

Also, a prospective laboratorial study, was conducted, where GBS isolates were serotypically characterized by molecular methods.

The results obtained were prepared as a manuscript that has been submitted for publication as an original paper.

Chapter 5 presents the general discussion and the main conclusions of the thesis.

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List of abbreviations

CAMP	Christie Atkinson Munch-Petersen
CDC	Centers for Disease Control and Prevention
CPS	Capsular polysaccharide
DNA	Deoxyribonucleic acid
EOD	Early Onset Disease
GBS	Group B *Streptococcus*
HIV	Human Immunodeficiency Virus
HPH	Hospital Pedro Hispano
IAP	Intrapartum Antibiotic Prophylaxis
LB	Live Births
LOD	Late Onset Disease
MLS	Macrolides, Lincosamides, Streptogramines
NT	Non-typeable
PCR	Polymerase Chain Reaction
PI	Pilus Islands
rRNA	Ribosomal RNA

Chapter 1

General Introduction

Chapter 1 – General introduction

1.1. Group B *Streptococcus*

1.1.1. History

In 1887, Norcard and Mollereau identified Group B *Streptococcus* (GBS) as a cause of bovine mastitis (inflammation of the mammary glands) [1]. In the early 1930s, based on a carbohydrate found on the bacterial cell surface, Rebecca Lancefield divided streptococcal bacteria into groups: group A (infections of humans); group B (infections of cattle); group C (affected various animals); group D (cheese); group E (milk) [2,3]. Shortly after, the English researchers Ronald Hare and Leonard Colebrook showed that these bacteria could be isolated from the genital tract of 10 % of women [4].

GBS was recognized as an etiological agent of severe human infections among neonates in the 1960s. Later, in 1973, studies ended up showing a link between GBS and infant mortality [5].

1.1.2. Characterization

The genus *Streptococcus* can be classified into Lancefield groups (Groups A–H and K-V) [6]. All Lancefield groups were assigned to one or more species, for example: Group A (*Streptococcus pyogenes*), Group B (*Streptococcus agalactiae*), Group C (*Streptococcus equi* ssp. *equi* and *Streptococcus dysgalactiae* ssp. *dysgalactiae*) and, more recently, Group M (*Streptococcus fryi*) [6].

GBS, also known as *Streptococcus agalactiae*, is a commensal that colonizes the gastrointestinal and genitourinary tracts of healthy adults, particularly in women [7,8].

Streptococcus agalactiae is catalase negative, Gram positive cocci bacterial species, which characteristically occurs in pairs or small chains (Figure 1.1.) [9-12].

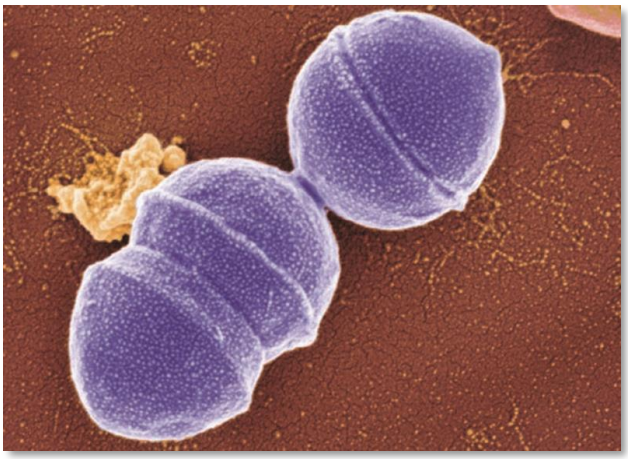

Figure 1.1. – Group B *Streptococcus* or *Streptococcus agalactiae* scanning electron microscopic image [5].

Metabolically, GBS is a facultative anaerobe and β-hemolytic. GBS shares a common antigen, the Lancefield Group B polysaccharide antigen (with repeated units of rhamnose). To distinguish GBS strains, type-specific capsular polysaccharides (CPS) are used. CPS are essential for the virulence of GBS and currently can be differentiated into 10 structurally and antigenically unique serotypes (Ia, Ib, and II to IX). Their distribution varies worldwide [7,13,14].

1.1.3. Colonization and transmission

GBS affects pregnant women, non-pregnant immunosuppressed patients (with diabetes mellitus, cancer, liver cirrhosis, human immunodeficiency virus (HIV) and other immune compromised states) and elderly individuals, these with an estimated mortality of 50 % [10,15]. In pregnant women, the disease appears typically as an infection of the genital tract, placenta or amniotic sac, or as bacteremia with either a genitourinary or an unknown source. Vertical transmission of GBS can occur in the uterus during labor and birthing, which means that pregnant women who carry GBS in the genital tract have a higher probability of infecting the neonates (Figure 1.2.).

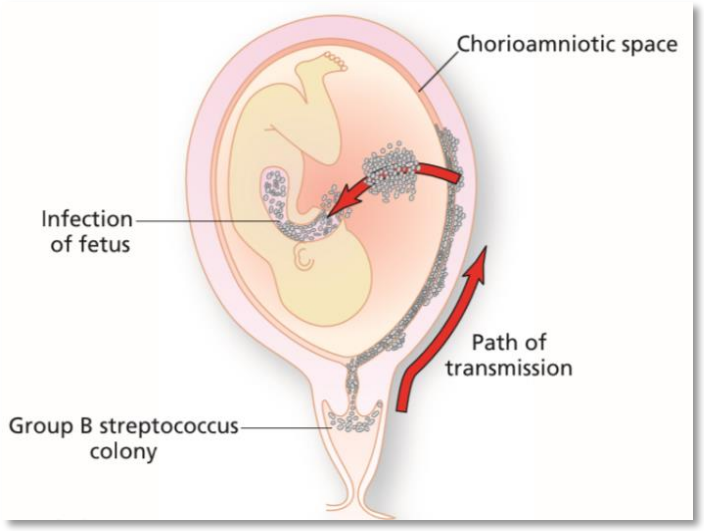

Figure 1.2. – Vertical transmission of GBS infection [5].

This transmission occurs in 15 to 50 % of newborns from GBS colonized mothers, with an estimated range of 5 to 40 % worldwide. Of these newborns 2 % may develop GBS infection [9,10,13,16-18].

Maternal and neonatal GBS colonization rates have been found to vary between different countries, different ethnic groups, gestational age and laboratory procedures on the sampling [8,19]. The prevalence of GBS vaginal colonization among pregnant women varies between 10 to 30 % [19,20].

1.2. Group B streptococcal disease

Due to its genitourinary tract location GBS is the major cause of early onset GBS disease (EOD - within the first 6 days of life) and late onset disease (LOD - between 1 week and 3 months of age) in newborns with mortality rates up to 10 %. Septicemia and pneumonia are the most common problems for EOD and meningitis for both EOD and LOD. For 25 to 35 % of the surviving infants that faced meningitis, they have high probability to have permanent neurological sequels (including sight or hearing loss and mental retardation) [21,22].

Early onset disease can result of vertical transmission from a colonized mother during or just before delivery. Late onset disease, is less well understood, however, can be acquired by vertical transmission from the mother or from nosocomially and community acquired infection [23]. Also, LOD is less often fatal and differs from EOD in terms of Group B *Streptococcus* serotype (sepsis associated with serotype III), clinical

manifestations and outcome [24]. A mortality rate of 14 % with EOD compared with 4 % of LOD has been shown in an observational study [25].

Lethality from both early onset and late onset disease is high [23]. The average incidence of EOD was found to be 0.43 per 1000 live births (LB). United States has an estimated incidence of 0.77 to 1 per 1000 LB of early onset neonatal sepsis [26], Africa 0.53 per 1000 LB, Americas 0.50 per 1000 LB and Europe 0.45 per 1000 LB. The lowest incidence rate found by Edmond et al. [23] was in South East Asia with 0.11 per 1000 LB. The average incidence of LOD was found to be 0.24 per 1000 LB with the highest rate in Africa with 0.71 per 1000 LB followed by the Americas with 0.31 per 1000 LB [23].

1.2.1. Global incidence of GBS Infection

Recent analyses, based on published studies, have reported a mean global incidence of GBS of 0.53 per 1000 LB [27].

Overall, the incidence of GBS infection ranges from 0.17 to 3.06 per 1000 LB in developing countries [12] and the mortality rate in these countries is higher than in developed countries (10-60 % compared to 7-11 %) [28]. In South Africa the HIV infection between pregnant women is high which can aggravate the problem of GBS disease [28]. In Europe the incidence is 0.5 to 2 cases per 1000 LB with a mortality rate of 4 to 10 % [13].

1.3. GBS Screening

GBS colonization can change over the progress of a pregnancy (can be transient, intermittent, or persistent), therefore it is important to do a specimen collection for determination of the colonization status [29].

Normally the culture-based screening methods rely on the collection of a vaginal/rectal swab specimen demonstrating a significantly higher yield than collection of vaginal samples alone [30]. Also, GBS culture swab taken from a neonate's umbilicus, throat, and ear after birth is used to determine positive or negative neonatal colonization. Depending on the number of sites that are sampled and analyzed, the rate of colonization may be influenced. Sensini et al. [31] has demonstrated that samples from neonate's umbilicus, throat and ear yielded higher isolation rates, 47.2 % compared to 23.2 % when only one site was sampled. Furthermore, these authors have shown that the rates of vertical transmission varied upon colonization density (high density of colonization (50.0 %) and lower density of colonization (30.4 %)) [31].

Detection culture medium and specimen delivery are two important factors for the obtainment of the best results regarding GBS colonization. Traditionally, sheep blood agar (SBA) and Columbia colistin-nalidixic agar (CNA) are used, even though both underestimate the incidence of GBS and require up to 48 hours for identification. Being aware of that, the Centers for Disease Control and Prevention (CDC) recommends the use of a selective broth like LIM broth or carrot broth (SCB), as they improve substantially the GBS detection. These culture methods are expensive and time-consuming and an evaluation of current methods is important to found a sensitive, cost effective and time efficient detection test which would allow early diagnosis and a more efficient prevention program [12].

Tests such as Gram stain, Christie Atkinson Munch-Petersen (CAMP) test or typing via an agglutinin reaction on selected β-hemolytic colonies are used to confirm the presence of GBS [32]. When grown on blood agar, GBS exhibit a β-hemolytic (lysis of red blood cells) appearance producing a clear zone around colonies (zones of hemolysis with 1-2 mm in diameter) [5] (Figure 1.3.A). GBS is the only β-hemolytic *Streptococcus* that secretes a protein called CAMP factor or "protein B". For this reason, CAMP test is used for identification of GBS [15]. For this test, *Staphylococcus aureus* that produces a lysin (β-lysin) is streaked across a blood agar plate. Then, strains of GBS are streaked on the same plate, perpendicular to *S. aureus*. Since lysin produced by *S. aureus* only lyses red blood cells, CAMP factor will react with this lysed area of the blood agar plate, enhancing the hemolytic activity [5,29]. Figure 1.3.B illustrates an arrowhead shape of the zone of enhanced hemolytic activity by the GBS near to the *Staphylococcus* streak [29].

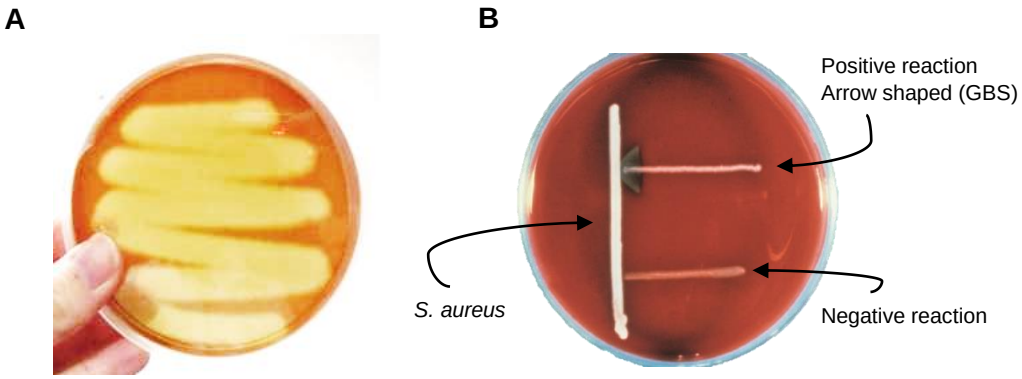

Figure 1.3. – A – Hemolyze; B – CAMP test. Adapted from [5].

GBS antigen detection compared to standard culture method is rapid, sensitive, specific and simple, as it only requires LIM broth, an incubator, antigen detection kit, minimal technician training and it is very easy to adapt to small laboratories [33]. This detection is done by a GBS antigen latex agglutination test that has been demonstrated to be reproducible. The result is considered positive if at least two agglutination reactions occurs within the 60s period [27,33].

Molecular biology-based assays, such as Polymerase Chain Reaction (PCR), can detect GBS, from vaginal samples in pregnant women in labor, more rapidly and reliably than the standard culture-based method. In fact, values of colonization rate assessed by culture methods are lower than those by PCR [18]. Some studies identify PCR as an accurate method since it is the only method with a 1 to 2 h turnaround time and has been approved by the Food and Drug Administration (FDA) for the detection of GBS DNA directly from vagino-rectal specimens [33]. Also, it is a technique with which the majority of the negative results are truly negative, while the positive ones should be confirmed by other methods such as culture or serology. However, strain culture would still be necessary to provide antimicrobial susceptibility information, especially in penicillin allergic patients [18,30,32].

PCR-based assays allow GBS detection in preterm pregnant women, women without antenatal care or without antenatal culture performed or women without GBS result at delivery [34].

1.4. Prevention and treatment

1.4.1. Centers for Disease Control (CDC) and Prevention 2010 Guidelines

GBS screening coupled with intrapartum antibiotic prophylaxis (IAP) has shown to decrease the incidence of invasive GBS on early-onset group B *Streptococcus* infections and, at the same time, prevent neonatal transmission of GBS. Thus, CDC 2010 Guidelines for Prevention of Perinatal Group B Streptococcal Disease, endorsed by the American Academy of Pediatrics, the American Academy of Family Physicians, and the American College of Obstetricians and Gynecologists recommended the screening of women between 35 and 37 weeks of pregnancy to determine GBS carrier status and prophylactic antibiotic treatment before labor for women at risk of transmitting this pathogenic. Other strategy suggested by CDC guidelines consists in identifying important maternal risk factors such as previous infant with invasive GBS disease, GBS bacteriuria during any trimester of the current pregnancy, positive GBS vaginal-rectal screening culture in late gestation during current pregnancy, unknown

GBS status at the onset of labor and any of the following factors: delivery at < 37 weeks gestation, amniotic membrane rupture ≥ 18 hours, intrapartum temperature ≥ 38.0 °C, intrapartum NAAT (Nucleic Acid Amplification Tests, such as Polymerase Chain Reaction (PCR)) positive for GBS [8,29,35].

The recommended screening and prophylaxis strategies by CDC have been very successful, reducing the incidence of early onset GBS by more than 80 % [36]. In 1990, the incidence in USA was 1.4 cases per 1000 LB but these values declined to 0.34 per 1000 LB after the implementation of the guidelines recommended by the CDC and the practice of antibiotic prophylaxis [13,17].

1.4.2. Antibiotic choices

Clinical trials demonstrate the efficiency of both penicillin and ampicillin as intravenously administered intrapartum agents for the prevention of EOD. GBS appears to be susceptible to penicillin, thus it is the first drug of choice for the prophylaxis and treatment [37]. Also, 2010 CDC guidelines recommend intrapartum antibiotic prophylaxis for prevention of EOD (Figure 1.4.).

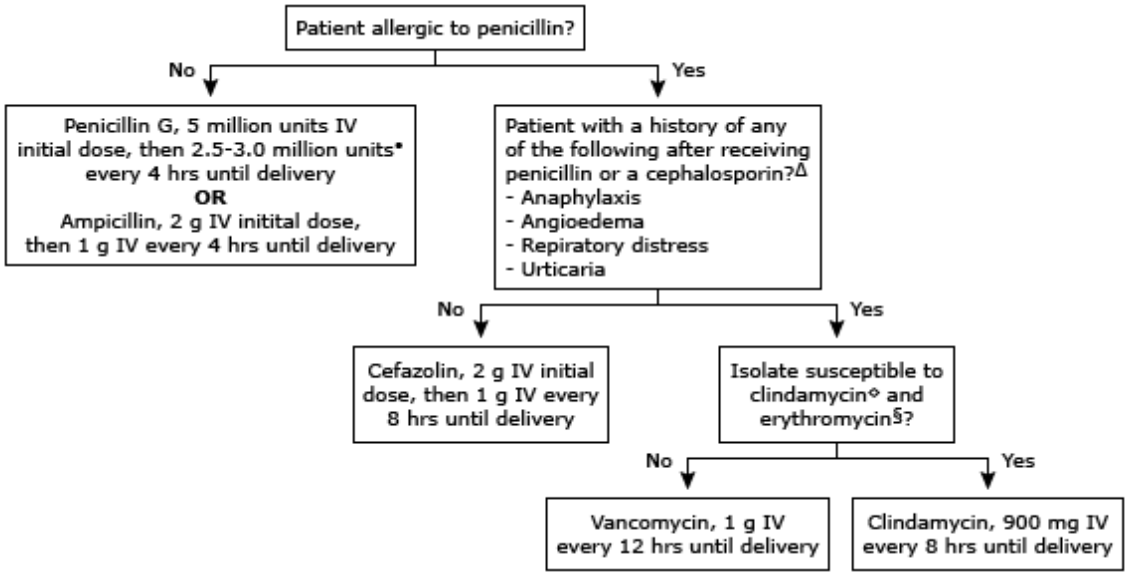

Figure 1.4. - Recommended intrapartum antibiotic prophylaxis for prevention of EOD [29].

Penicillin G has a narrow spectrum of antimicrobial activity which reduces the risk of selective pressure on other organisms and decreases the risk of ampicillin resistance. The dosages administrated of penicillin and ampicillin for IAP intended to

achieve adequate levels in the fetal circulation and amniotic fluid rapidly, avoiding neurotoxic serum levels in mother or fetus [29].

In general, it has been found that β-lactam antibiotics for GBS are highly effective at preventing vertical transmission when intravenously administrated for ≥ 4 hours before delivery. Shorter durations might provide some protection (≥ 2 hours before delivery). Oral antibiotic therapy does not show satisfactory rates of clearance of GBS from the genital tract in the time of labor, so they are not adequated for GBS prophylaxis [29, 38].

In the case of penicillin-allergic women, who are at low risk for anaphylaxis, cefazolin is recommended, which has a relatively narrow spectrum of action. However, it is estimated that 10 % of persons with penicillin allergy also have hypersensitivity reactions to cephalosporins. For women who are at high risk for anaphylaxis from penicillin, 2010 CDC guidelines also recommend intravenous vancomycin and clindamycin [29].

Other options for penicillin-allergic women are macrolides or lincosamides [37]. These structurally unrelated antimicrobials are grouped into a single family, the macrolides, lincosamides and streptogramines (MLS) family. This classification is justified by a similar, although not identical, mechanism of action [40]. This class of antibiotics includes erythromycin and clindamycin that bind into the 50S subunit of the ribosome and attach to their subunit 23S rRNA. They inhibit protein synthesis by interfering with formation of initiation complexes for peptide chain synthesis or may interfere with aminoacyl translocation reactions. Resistance to macrolides can be acquired from an alteration (methylation) of the rRNA receptor and can occur mainly by two mechanisms: modification by methylation of the antibiotic target and the active efflux of the antibiotic across the membrane. A methylase, encoded by the *erm* gene, does the target site modification, which leads to the inducible or constitutive expression of resistance phenotype (iMLSB and cMLSB respectively) [8,15,41].

GBS resistance to macrolide antibiotics has been increasing in Europe and the USA [15]. In the USA the resistance values to erythromycin vary from 7 to 21 % and to clindamycin from 3 to 15 % [8].

Alternatives to penicillin and ampicillin, such as cefazolin, clindamycin, erythromycin and vancomycin have not been measured in controlled trials [29].

1.5. Vaccination

A new improving method is under study to prevent GBS in pregnant women. Maternal vaccination represents the most attractive strategy.

Effective vaccines aimed to reduce maternal colonization and to stimulate the production of anti-GBS antibodies enhancing transplacental transfer to the fetus. It has been demonstrated an association between high maternal serotype-specific anti-capsular polysaccharide antibody concentrations with reduced risk of recto-vaginal colonization and reduced risk of newborns developing EOD [42].

It is known that virulence factors facilitate the adhesion to and invasion of the host cells, as well as in the evasion from the immune system. GBS has a variety of these virulence determinants and some have been identified and characterized [43]. The most promising vaccine candidates include CPS and surface proteins, including the pilus islands (PI) proteins [14].

1.5.1. Specific Capsular Polysaccharides

CPS are important antiphagocytic virulence factors and contributes to GBS survival *in vivo* by avoiding recognition by the host defense system [22,44]. The structure of the capsular polysaccharide is determined by locus *cps* genes, that encode enzymes responsible for the synthesis of the polysaccharides, and is composed of 16-18 genes [45]. Ten CPS types, with chemical and antigenic differences, have been described (Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX) (Figure 1.5.) [43].

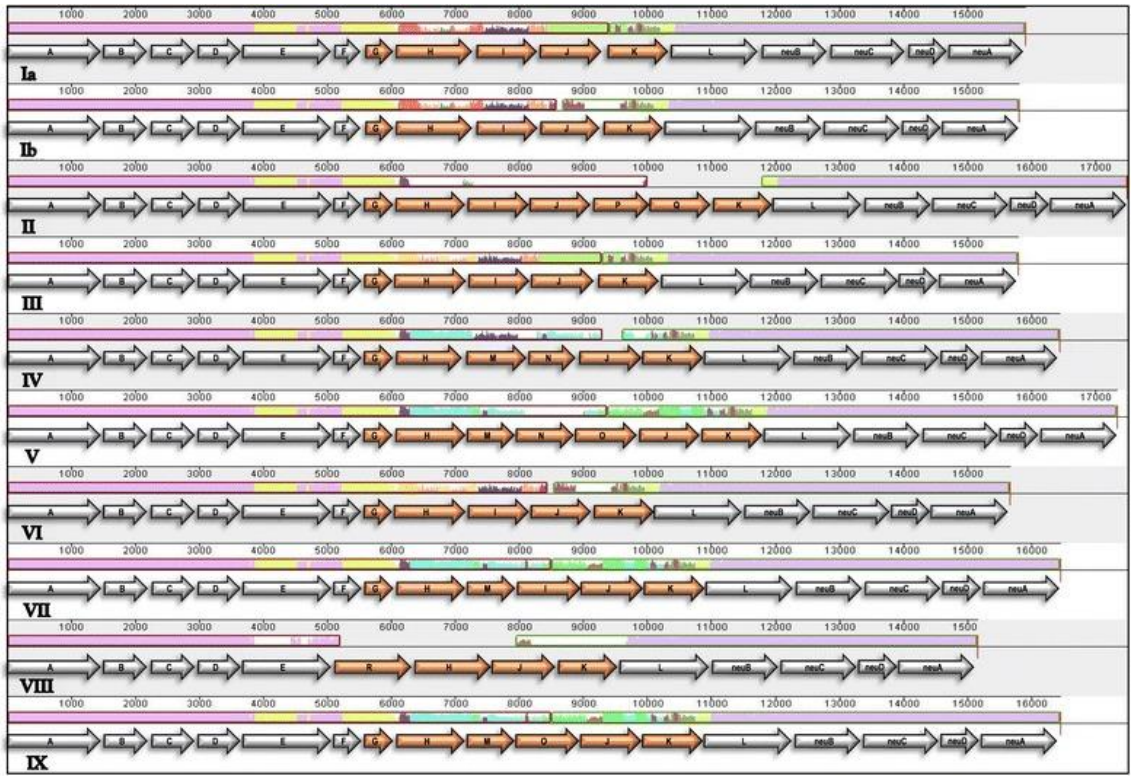

Figure 1.5. – Comparison of the *cps* loci of all 10 serotypes (Ia, Ib, III-IX). [45].

They are mainly composed of repeating units containing four elements: glucose, galactose, N-acetylglucosamine and sialic acid [41].

Most cases of EOD in newborns are caused by serotype Ia, serotype III is responsible for LOD and serotype V for infections in adults, mostly in the elderly or immunocompromised patients [41]. There are also major differences between countries in the distribution of GBS serotypes [44].

Vaccine candidates against Ia, Ib and III serotypes are in development and have been evaluated for safety and immunogenicity [16, 27]. Also, a trivalent vaccine, including serotypes Ia, Ib and III, conjugated to mutated *Corynebacterium diphtheria* toxin (CRM_{197}) is under clinical evaluation [14,16]. CRM_{197} is nontoxic, it has more lysyl side chains available for conjugation (compared to wildtype toxin) and it has a great versatility for conjugation to multiple polysaccharides in the same product. In fact, CRM_{197} was used in some licensed conjugate vaccines against *Neisseria meningitidis* and *Streptococcus pneumoniae* [14].

1.5.2. Pilus Islands (PI) proteins

Surface protein antigens such as pilus island proteins, present in all GBS isolates, can incorporate other potential immunization candidate, as it plays a major role in bacterial adhesion to the uro-genital mucosa [42,46].

Pili are filamentous structures extending out from the bacterial surface visible by electron microscopy [14] and they are composed by a backbone protein (*bona fide* pilin) and two ancillary proteins (AP1 and AP2), a pilus associated adhesin and a component that anchors the pili to the cell wall [41].

GBS pili genes are located within two distinct loci in different regions of the genome, pilus-islands 1 and 2 (PI-1 and PI-2), later representing two distinct variants, PI-2a and PI-2b [41]. Pili are important in colonization and invasion of the respiratory, urinary and intestinal tracts.

Normally pili are associated with Gram negative bacteria such as *Escherichia coli* and *Neisseria* species [47,48]. But have also been described in Gram positive organisms [49-51].

1.5.3. Serotyping

To decide vaccine formulations, it is important to identify the clinical and molecular epidemiology of GBS serotypes, pilus island and genotypes and the invasive potential of different serotypes and strains [46].

Tracking the serotypes associated with GBS cases, in different regions, is important, since multivalent vaccines are being developed against the serotypes known to cause most invasive cases [52]. With this data, it will be possible to optimize the coverage against invasive GBS disease. However, there is a lack of data on serotypes and genetic diversity in industrializing countries and no information on pilus-island distribution [7,13,46].

Molecular biology-based assays have shown to be consistent and more rapid than the standard culture-based method. Therefore, PCR assays for antigen detection have been evaluated in terms of sensitivity and specificity. Also, the colonization rate by traditional culture methods is low when compared to antigen detection method and strain identification by 16S rRNA PCR analysis [18].

1.6. Portuguese reality

The Portuguese Pediatric Surveillance Unit-Portuguese Society of Pediatrics began GBS registration in 2001 and showed an incidence of GBS in Portugal of 0.54 per 1000 LB and a mortality rate that varies between 4 and 6 % in term neonates, being higher in preterm infants [53]. Later, in 2004, the National Consensus on Neonatology was published, suggesting a uniform national action protocol [54,55].

Few national published research articles about this subject exist.

In 2008, Maria Teresa Neto from neonatal intensive care unit (NICU) from Hospital de Dona Estefânia, Lisboa, has reported a wide range in the incidence of GBS, according to geographic areas (North 0.9/ 1000 LB; Centre 0.4/ 1000 LB; Lisbon and Tagus Valley 0.4/ 1000 LB; Algarve 0.1/ 1000 LB and Islands 0.2/ 1000 LB) [53]. However, the overall incidence of GBS disease was 0.54 per 1000 LB which can demonstrate that even in a small country the incidence varies from one region to another. A decreased incidence has occurred between 2002 and 2004 (2002 – 0.60/ 1000 LB, 2003 – 0.58/ 1000 LB and 2004 – 0.38/ 1000 LB), which may have been due to the emergent screening and prophylaxis strategies. The mortality rate for Portugal reported in this study was 6.6 % which was in the range of other countries from Europe: UK 9.7 % and Germany 4.3 % [53,56].

Another study published in 2010, reported that at the Hospital São Marcos, Braga, the prevalence of colonized pregnant women was 34.9 % [55]. The prevalence of neonatal GBS infection was 9 per 1000 LB, a much higher value than the one reported by Unidade de Vigilância Pediátrica (UVP) da Sociedade Portuguesa de Pediatria in 2001 which indicated a prevalence of 0.5 per 1000 LB [55].

More recently, in 2014, a study performed between 2005 and 2012 showed that the most common GBS serotypes were Ia, II, III and V (serotype III (23 %), Ia (21 %), V (20 %), II (14 %)), but with random distribution over the years [57]. This serotype prevalence is similar to that found in 2002 by Coelho et al. [58] (serotype III (24.6 %), V (23.4 %), Ia (17.8 %) and II (16.3 %)). Also, Florindo and collaborators have reported an emergence of serotype IV creating a real concern about the antibiotic resistance due to their co-resistance to second-line macrolide antibiotics and their virulence potential [57].

As epidemiological studies of GBS in Portugal are still limited, it is important to conduct further studies in order to amplify the knowledge of this public health problem.

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Chapter 2

Objectives and workplan

Chapter 2 – Objectives and workplan

Even though prevention and intervention strategies have been developed, GBS remains a public health issue being the principal cause of neonatal infections. For this reason and because these strategies are directly influencing the neonatal mortality, the main purpose of this work was to analyze different aspects of the GBS prevalence and distribution.

As this burden disease varies between countries, an epidemiologic surveillance is imperative. In this thesis a bibliographic review on the GBS status in Europe, suggested by Professor Olga Lage (FCUP, Department of Biology), was developed. The data obtained helped on identifying the GBS incidence at European level and discover (1) the differences between the procedures/ methodologies used (screening, culturing and antibiotic prophylaxis), (2) the maternal GBS colonization, (3) the serotype distribution and (4) antimicrobial resistances in different European countries. This study was based on recently published articles and some older ones for comparison, using reference databases such as NCBI (PubMed). The purpose of this study was to do the state of the art at European level on the GBS epidemiology and screening methods and try to show the importance of implementing fixed and universal guidelines in order to see some methodological errors minimized. All the information obtained was gathered into a review paper (Chapter 3).

Matosinhos is a municipality belonging to the metropolitan area of Porto and is constituted by: União de Freguesias de Lavra, Perafita e Santa Cruz do Bispo, União de Freguesias de São Mamede Infesta e Senhora da Hora e União de Freguesias de Custóias, Guifões e Leça do Balio. Hospital Pedro Hispano located at Senhora da Hora is a reference hospital providing care to the population residing in Matosinhos, about 175 000 residents. Furthermore, it is a direct reference hospital for the users coming from Vila do Conde and Póvoa de Varzim, reason why it provides care to a total of 318 000 users. In 2013 the birth rate was 7.8 LB per 1000 habitants and the infant mortality rate was 3.6 cases per 1000 LB. In December 2015, a total of 1457 deliveries was registered [1].

Together with Dr. José Aires Pereira (Pediatric and Neonatology Service) and Dr. Valquíria Alves (Clinical Pathology Service), several work themes were discussed and the GBS infections in pregnant women and neonates were highlighted by its worldwide health importance. The local incidence of GBS and the serotyping of the microorganism, in order to perceive the most common serotype in the locality, were two

topics discussed with the final objective to raise awareness of this public health problem and the prospective implementation of a vaccine, still under study. Thus, Dr. José Aires Pereira was the hospital supervisor of the work and provided information from the Neonatology service; Dr. Valquíria Alves provided laboratory information on the screening requests for GBS and clinical GBS samples and Dr. António Araújo (Biogerm) provided the facilities and the culture medium to initiate the cultures for GBS serotyping.

A local study was performed focusing on the incidence of *Streptococcus agalactiae* in pregnant women between 35 and 37 weeks of gestation and in hospitalized neonates from pregnant women between 24-41 weeks of gestation between 2011 and 2016. Therefore, with GBS screening information from pregnant women between 35 and 37 weeks of gestation provided by Dr. Valquíria Alves analyses were performed to statically understand (1) how many GBS screenings from pregnant women were requested, (2) how many were colonized with GBS, (3) the prevalence between age groups and (4) the incidence variation over time. The data provided included information about the sample, product entry, patient's age, clinical information and positive or negative results for GBS. Dr. José Aires Pereira provided Neonatology clinical files of hospitalized neonates from pregnant women between 24-41 weeks of gestation being possible to analyze (1) the total hospitalized neonates, (2) the gestation information (gestational age, type of delivery, neonate weight, neonate gender, reason for hospitalization and diagnosis), (3) the colonization GBS status, (4) the prevalence between age groups and (5) the prophylaxis procedures performed.

Since an innovative method of vaccination is being studied, it is important to identify the serotype distribution of GBS. For this reason, a statical study on the GBS serotypes distribution was performed. Sixty-seven clinical *Streptococcus agalactiae* isolates (kept at -80 ºC) were provided by Dr. Valquíria Alves (Clinical Pathology Laboratory) for this work. These samples were cultured on selective medium plates (Granada Agar at 36 °C for 24-48 h) provided by Dr. António Araújo and grown at Biogerm facilities.

Serotyping was conducted by a multiplex PCR method. This technique uses specific primers for the amplification of the genes of the different serotypes, as described by Poyart et al. [2]. This process was held at Sciences Faculty facilities under the supervision of Professor Olga Lage and the results were presented in percentages and compared with the values of other studies carried out in Portugal, namely the recent study of Florindo et al. [3].

The results obtained from hospital information and serotyping were gathered in an original article (Chapter 4).

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Chapter 3

Incidence and distribution of the invasive *Streptococcus agalactiae* in European countries

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The results obtained in this chapter were presented to elements belonging to the Department of Neonatology and Clinical Pathology of Hospital Pedro Hispano.

Incidence and distribution of the invasive *Streptococcus agalactiae* in European countries

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Abstract

Background: *Streptococcus agalactiae*, commonly referred as Group B *Streptococcus* (GBS), has been recognized as a worldwide causative pathogenic agent of neonatal sepsis, meningitis and pneumonia. As this burden disease varies between countries, an epidemiologic surveillance is imperative which is the focus of this review.

Materials and methods: To better understand the GBS behavior and incidence, an European study based on published literature was performed. Based on a comprehensive bibliographic search studies containing data such as rates of GBS carriers, serotype distribution and antimicrobial resistances were considered.

Results: A total of sixty-five studies presented data from 20 European countries. The rates of GBS carriers ranged from 6.5 % to 36 %. The most common serotypes reported among the studied countries were serotype III with an approximate mean value of 37 % followed by serotype Ia with 18 %. Due to emerging GBS resistance, the two antibiotic alternatives to penicillin used in cases of allergy, erythromycin and clindamycin, are becoming problematic.

Conclusions: GBS continues to be a major public health problem, present at high rates in some European countries. Further scientific work on the epidemiology of GBS has to be carried out for a better monitorization and development of common methodologies aiming to prevent this burden disease.

Keywords

Group B *Streptococcus*, Pregnant women, Bacterial colonization, Neonatal sepsis, Intrapartum antibiotic prophylaxis.

Introduction

Streptococcus agalactiae, also known as GBS, is a normal commensal that colonizes the gastrointestinal and genitourinary tracts of healthy adults, particularly in women [1,2]. GBS members share a common antigen, the Lancefield Group B polysaccharide antigen, and are distinguished by their type-specific capsular polysaccharides (CPS). CPS are essential for GBS virulence and currently can be differentiated into 10 different structurally and antigenically unique serotypes (Ia, Ib, and II to IX) and their distribution varies worldwide [1,3,4].

Although GBS are normal commensals of gastrointestinal and genitourinary tracts, they are the major cause of early onset disease (EOD - within the first 6 days of life) and late onset disease (LOD - between 1 week and 3 months of age) in newborns with mortality rates up to 10 % [5,6]. It also affects pregnant women, non-pregnant immunosuppressed patients (with diabetes mellitus, cancer, liver cirrhosis, HIV and other immune compromised states) and elderly individuals, these with the estimated mortality of 50 % [7,8]. Maternal and neonatal GBS colonization rates have also been found to vary between different countries, different ethnic groups, gestational age and laboratory procedures on the sampling [2,9].

In 1990, the incidence in USA was 1.4 cases per 1000 livebirths (LB) but these values declined to 0.34 per 1000 LB after the implementation of guidelines recommended by Centers for Disease Control and Prevention (CDC) and the practice of antibiotic prophylaxis [3,10]. GBS screening coupled with intrapartum antibiotic prophylaxis (IAP) has shown to decrease the incidence of invasive GBS on early-onset Group B *Streptococcus* infections and, at the same time, prevent neonatal transmission of the bacterium. Thus, CDC 2010 Guidelines for Prevention of Perinatal Group B Streptococcal Disease recommended the screening of women between 35 and 37 weeks of pregnancy to determine GBS carrier status and prophylactic antibiotic treatment during labor for women at risk of transmitting this pathogenic to the newborn. Other strategy suggested by CDC guidelines consists in identifying important maternal risk factors such as previous infant with invasive GBS disease, GBS bacteriuria during any trimester of the current pregnancy, positive GBS vaginal-rectal screening culture in late gestation during current pregnancy, unknown GBS status at the onset of labor (culture not done, incomplete, or results unknown) and

any of the following: delivery at < 37 weeks gestation, amniotic membrane rupture ≥ 18 hours, intrapartum temperature ≥ 38 °C, intrapartum NAAT (Nucleic acid amplification tests) positive for GBS [2,11,12]. Despite the prevention, *S. agalactiae* is still an important cause of severe infections. Its potential emergent tolerance to the drug of choice, the penicillin, and the resistance to clindamycin and erythromycin, used in patients with a history of beta-lactams allergy, has raised concern [8,11,13].

Recent analyses based on published studies have reported a mean global incidence of GBS of 0.53 per 1000 LB [14] and 0.5 to 2 cases per 1000 LB in Europe with a mortality rate of 4 to 10 % [3].

A systematic review of published literature that assessed the rate of GBS carriers, serotype distribution of GBS strains and their susceptibility to antibiotics in European countries is the aim of this work. These data can subsequently be used to identify similarities or differences between countries on the methodologies used and the results obtained. Additionally, it will help and guide public health initiatives to reduce the prevalence and burden of this infection.

Materials and methods

Search Strategy

Electronic datasets were used for this study, including NCBI (PubMed), ScienceDirect, ResearchGate, some online journals such as The Lancet, Oxford Journals (The Journal of Infectious Diseases, http://jid.oxfordjournals.org, Journal of Antimicrobial Chemotherapy, http://jac.oxfordjournals.org), Microbiology Society (Journal of Medical Microbiology, http://jmm.microbiologyresearch.org) and online sites such as Centers for Disease Control and Prevention (www.cdc.gov). The main keywords used in the search strategy were: "*Streptococcus*", "*Streptococcus agalactiae*", "Group B *Streptococcus*", "Group B streptococcal disease", "Group B *Streptococcus* colonization", "GBS", "Neonatal infections", "Infant infection" and "GBS epidemiology".

Data was extracted to allow descriptive analyses based on study design, location, study period, sample size, sampling method, maternal colonization, serotype distribution and antibiotic susceptibility.

Study inclusion and exclusion criteria

Initially all articles written in English were considered. The documents retrieved from this search were later reviewed by title and abstract to include only those that met the criteria for inclusion of research. Relevant studies included information such as

maternal colonization, serotype distribution, antibiotic susceptibility and the study design/methodology used in European countries. No restrictions were placed on study design and sampling methods. Each study was analyzed and organized according to the contained information. Studies that did not evaluate relevant outcomes to this study or that were not representative of European countries were not included for analysis.

Selection of studies

Out of the 233 studies encountered, 114 showed to have European information with relevant title and abstract. Figure 3.1. describes the results of the search process and the application of the inclusion and exclusion criteria, resulting at the end in 65 studies. These were considered if they included all or at least one of the referred stated criterion.

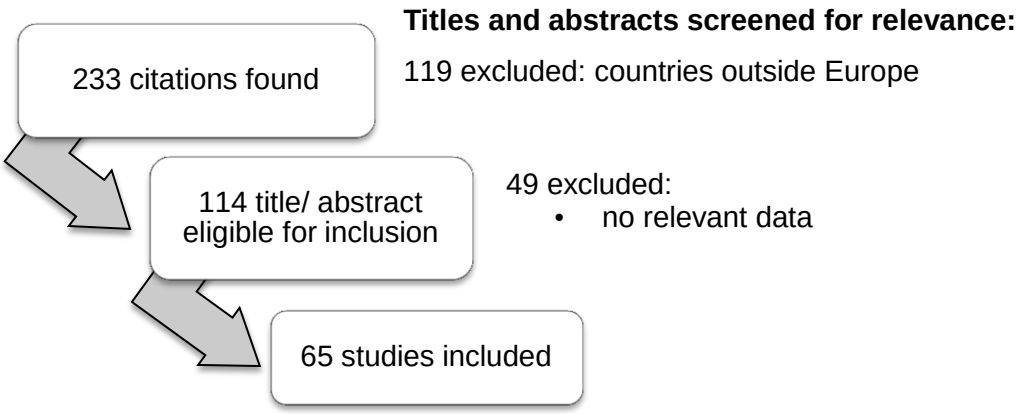


Figure 3.1. – Structure of the selection of the literature.

Results and Discussion

One hundred and fourteen scientific studies reporting data from Europe were found, of which only 65 fulfilled the information criteria. The criteria established that articles should contain data of at least one of these topics: rates of GBS carriers, serotype distribution and antibiotic resistances. Thirty-one articles (47.6 %) contained at least one, twenty-six (40 %) contained two and only eight (12.3 %) contained the three different types of information. The geographical distribution of the studies is represented in Figure 3.2.

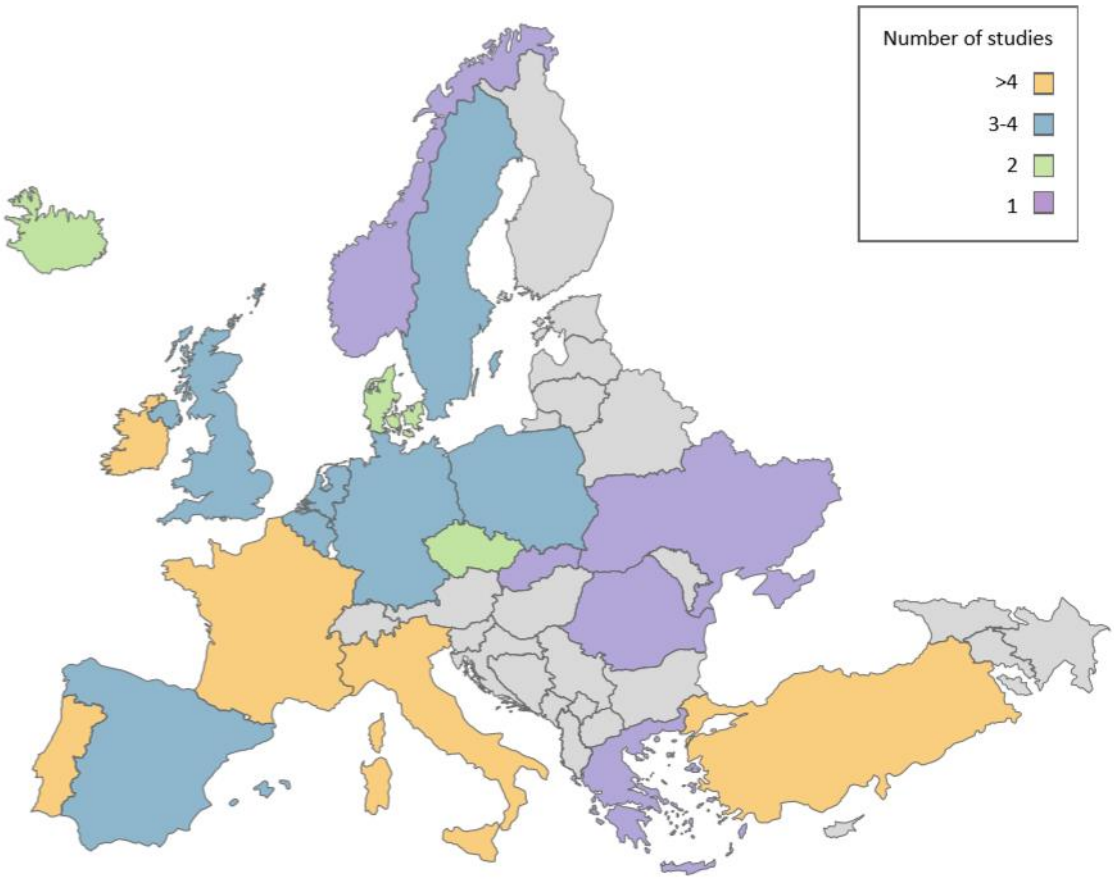


Figure 3.2. – Number of considered studies per European country.

Although data was recovered from 20 countries of the European area, the representativeness of some is low due to low number of published papers. In addition, there is a lack of information in both Northern Europe (e.g. Estonia, Finland, Lithuania) and Southern Central Europe (e.g. Croatia, Serbia, Slovenia).

Relevant facts on GBS studies

These 65 studies presented data on pregnant women, non-pregnant women and invasive infections on neonates. The majority of the studies (96.9 %) described the study time period, 53.8 % provided information about culture methods and 67.7 % about GBS identification methods. Moreover, 33 studies assessed the rates of GBS carriers, 41 GBS serotype distribution and 30 evaluated antimicrobial resistances. In 38.5 %, demonstration of knowledge or use of the recommendations of CDC guidelines were reported but other guidelines were also mentioned in other studies such as French Society for Microbiology standards (CA-SFM), Agence Nationale d'Accréditation en Santé (ANAES) and Deutsche Industrie Norm (DIN). The design,

period and characteristics of the studies [15-79] are summarized in Table 3.3. in the Supporting Information section.

In some studies, a vaginal and/or a rectal swab (34 papers/ 52.3 %) sampling is referred. However, sampling with rectovaginal (11 papers/ 16.9 %) swabs have demonstrated to be more reliable for the detection of GBS colonization, 18.5-51 % more sensitive than vaginal swabs alone [80,81]. This suggests that maternal rectal colonization is an important source of GBS acquisition in newborns [80].

For the isolation of *S. agalactiae*, CDC guidelines recommended the enrichment in selective broth media such as Todd-Hewitt (TH) broth with gentamicin (8 µg/ml) and nalidixic acid (15 µg/ml) or with colistin (10 µg/ml) and nalidixic acid (15 µg/ml) (Lim broth), followed by subculture onto tryptic soy agar with 5 % defibrinated sheep blood, Columbia agar with colistin and nalidixic acid, or a commercial chromogenic agar [11]. Among the 35 articles that provided information on cultural methods, the majority used an enrichment medium followed by a subculture in blood agar plates, in Columbia agar or other chromogenic medium.

El Aila et al. [30] used different chromogenic media (Group B *Streptococcus* differential agar (Becton Dickinson) and ChromID™ Strepto B agar (BioMérieux, Marcy l'Etoile, France)) and showed that the direct plating in Columbia agar and/or GBSDA allowed a rapid detection of GBS. However, this work confirmed that those chromogenic media were as sensitive and specific as the one, Lim broth, recommended by CDC followed by subculture onto non-chromogenic agar.

For specific identification of GBS, slide agglutination tests or tests that allowed the detection of GBS antigen (as genetic probe or fluorescent antibody) and Christie Atkinson Munch-Petersen (CAMP) test for identification should be performed [11]. All the 43 articles that provided information on the GBS identification methods used at least one of these tests, complemented by other methods of identification such as Gram test, catalase test, bile-aesculin test, tDNA-PCR assay [30,31] and 16S rRNA gene identification [31].

These methods of GBS isolation and identification are essential for an accurate and correct identification of the causal disease agent.

GBS carriers

Thirty-three (50.8 %) studies included and/or studied GBS colonization (Table 3.1.).

Table 3.1. – Rates of GBS carriers in Europe.

Country	Period	Sample size	Population	GBS carriers (%)
Belgium [31]	2007	150	Pregnant women	24
Belgium [30]	2009-2010	300	Pregnant women	22
Czech Republic [59]	2001-2002	586	Pregnant women	29.3
Denmark [40]	1999-2001	77	Pregnant women	36
France [63]	1994-1995	2454	Pregnant women	11
France [76]	1994-1996	3906	Pregnant women	14.3
France [43]	1997-1999	370	Pregnant women	15.4
France [64]	2006-2007	109	Pregnant women	11
France [45]	2007-2012	192	Neonatal invasive infection	29
Germany [23]	2001-2003	210	Pregnant and non-pregnant women	16
Germany [50]	2004	869	Pregnant women	21.1
Iceland [21]	1994-1997	280	Pregnant women	24.3
Ireland [48]	Not stated	504	Pregnant women	25.6
Ireland [78]	1999-2001	1308	Pregnant women	11.3
Ireland [47]	2004, 2006	2000	Women between 15 and 54 years	16.1
Italy [67]	1993-1995	2300	Pregnant women	11.3
Italy [25]	2002-2005	5020	Pregnant women	17.9
Italy [65]	2005-2006	400	Pregnant women	18
Italy [20]	2014	3988	Pregnant women	9.8
Netherlands [73]	2000-2002	1702	Pregnant women	21
Norway [22]	2009-2011	1739	Pregnant women	26
Poland [49]	2001-2002	1678	Pregnant women	19.7
Poland and Ukraine [68]	2010-2011	100	Pregnant women	17.1 and 17
Portugal [53]	2004-2006; 2006-2007; 2009	2639	Pregnant women	13.9

Table 3.1. – Rates of GBS carriers in Europe (continue).

Country	Period	Sample size	Population	GBS carriers (%)
Portugal [15]	2005	1523	Pregnant women	34.9
Slovakia [42]	2000-2003	754	Newborns	21.4
Sweden [39]	2005	400	Pregnant women	25.4
Turkey [16]	2000	310	Pregnant women	10.6
Turkey [32]	2000-2001	500	Pregnant women	9.2
Turkey [79]	2001-2002	200	Pregnant women	6.5
Turkey [17]	2002-2003	300	Pregnant women	8
Turkey [46]	2002-2003	150	Pregnant women	32
UK [44]	2001-2003	748	Pregnant women	21.3

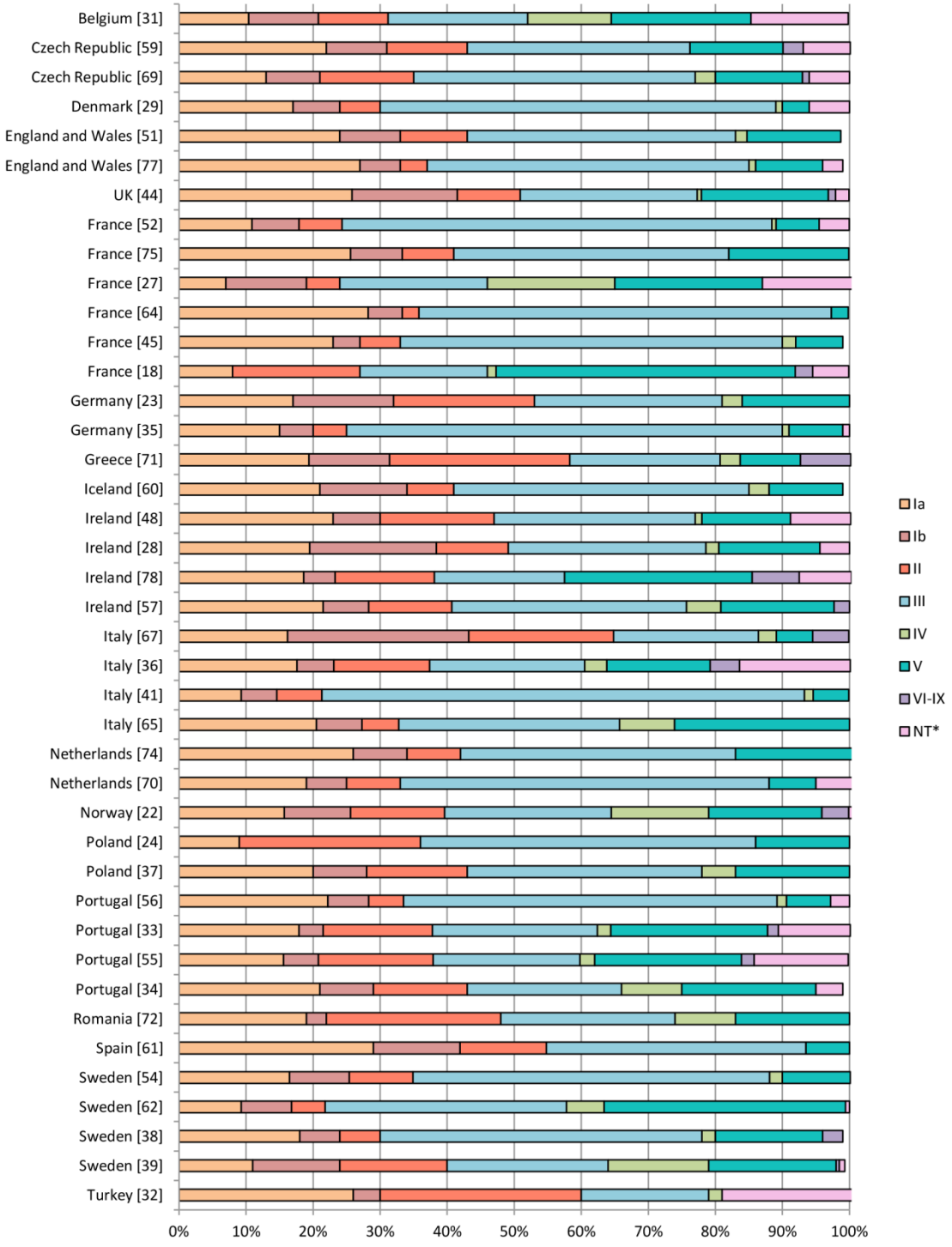
Of these studies, Denmark [40] and Portugal [15] showed a higher percentage of GBS carriers, 36 % and 34.9 %, respectively. It is clear that there is a variation between countries as rates ranged from 6.5 % to 36 % in the studies analyzed, with 22 of the 33 studies reporting rates ≥ 15 %. Women colonization varies among ethnic groups, geographical locations, gestational age and laboratory procedures on the sampling [2].

The ideal and recommended approach to collect and detect GBS carriage is swabbing, both the lower vagina and rectum, as late as possible in the pregnancy (usually 35-37 weeks' gestation) and cultivation in selective enrichment broths [11]. Therefore, in some of these studies the rates may have been underestimated due to poor methodology.

Curiously, Tsolia et al. [71] showed that women who were followed privately corresponded to higher colonization rates than those followed at a public inner-city hospital and who had poor prenatal care. This evidence contrasts with a study in which GBS colonization was often demonstrated in women of lower socioeconomic class followed at public hospitals [82].

Serotype distribution

Forty-one (63 %) studies included and/or studied the serotype distribution at European country level (Figure 3.3. and Table 3.4. on Supporting Information section).



*NT, Non-typeable.

Figure 3.3. – Serotype GBS distribution by country.

The most common serotypes among the 41 studies are serotype III, with an approximate mean value of 37 % followed by serotype Ia with 18 %. These serotypes are known to cause LOD and EOD in newborns, respectively [37]. In some European countries, serotypes Ia, Ib, and III have also been pointed to cause invasive disease in nonpregnant adults [83].

In general, serotype variations in each country were reported in different studies. Because of the rounding of the percentages values, some studies have not a total of 100 %. Also, rates from serotypes VI, VII, VIII and IX are shown together due to very small percentages. Percentage increases are recorded in two specific studies in England and Wales [51,77] and two studies from France [52,18] at different time intervals. Between the two studies from England and Wales [51,77] performed in 1995-2010 and 2000-2001, there was a 6 % increase in serotype II and 4 % in serotype V. Comparing the most recent and the oldest article from France [52,18], there was an increase from 6.41 % to 19 % of serotype II and from 6.41 % to 44.6 % of serotype V. The increase in serotype V may be associated with resistance to erythromycin. This serotype has emerged as a cause of human disease with high propensity to acquire macrolide resistance and spread [2]. Therefore, high concerns are raising about inadequate prophylaxis or treatment with these antibiotics [2]. It is also notable the increase of serotype III between two Portuguese studies from 21.9 % to 55.7 % [55,56] and between two other from Italy from 21.6 % to 72 % [41,67].

There is a variation in the incidence of serotypes between countries. The most striking ones are: Ia – 7 % France to 29 % Spain; Ib – 3 % Romania to 27 % Italy; II – 2.5 % France to 30 % Turkey; III – 19 % France and Turkey to 72 % Italy; IV - < 1 % UK to 19 % France; V - 2.5 % France to 36 % Sweden (although France has the higher rate of 44.6 %). These geographical differences between countries may be indicative of the existence of correlation to the different serotype incidences.

The methodologies performed for GBS characterization do not differ from what is currently advised by guidelines such as CDC. The kits using latex agglutination and analysis through PCR coupled to strain grouping techniques like MLST and PFGE stand out by being the most used [e.g. 18, 24, 27, 28, 29 and 83]. Other techniques such as precipitation reaction in gel and capillary tubes with antisera [59] and Lancefield acid extraction followed by gel immunoprecipitation [51] were also used.

Monitoring serotypes distribution is important to guide vaccination programs, which is a preventive strategy to protect the neonates from GBS infection, through mother's immunization.

Antibiotic resistance

Thirty (46.2 %) studies included and/or studied GBS antibiotic susceptibility at European country level.

The studies focused mainly on the analysis of antibiotics such as clindamycin, erythromycin, penicillin, tetracycline and vancomycin (Table 3.2.).

Table 3.2. – GBS antimicrobial resistance values (%).

Country	Year	Isolates (n.)	Clindamycin	Erythromycin	Penicillin	Tetracycline	Vancomycin
Belgium [26]	2001-2002	262	11.0	16.7	S[a]	NT[b]	S
Belgium [58]	2001-2003	187	10.7	19.2	S	NT	NT
Czech Republic [59]	2001-2002	586	3.2	3.8	S	83.9	NT
England and Wales [77]	2000-2001	353	NT	4	S	91	S
France [43]	1997-1999	370	NT	14	NT	89.5	NT
France [64]	2006-2007	109	NT	13.8	S	95.5	S
France [45]	2007-2012	169	10.7	16.7	S	89.8	S
France [18]	2011-2012	93	37.8	40	S	82.2	NT
Germany [66]	2001	338	4.7	11	S	NT	S
Germany [35]	2001-2003	296	5.7	10.1	S	NT	S
Germany [50]	2004	180	NT	11	S	NT	NT
Greece [71]	2000-2001	67	7.5	7.5	S	NT	S
Ireland [57]	2007-2011	177	NT	18.6	S	NT	S
Italy [36]	2002-2005	91	NT	16.5	NT	NT	NT
Italy [41]	2005-2008	87	NT	12	NT	93.3	NT

[a] Susceptible
[b] Not tested

Table 3.2. – GBS antimicrobial resistance values (%) (continue).

Country	Year	Isolates (n.)	Clindamycin	Erythromycin	Penicillin	Tetracycline	Vancomycin
Italy [65]	2005-2006	73	16.4	4.1	S	78.1	NT
Italy [20]	2014	902	NT	NT	NT	63.6	NT
Netherlands [70]	1997-1999	198	NT	S	S	NT	S
Norway [22]	2009-2011	423	9.6	10.2	S	76.6	NT
Poland [24]	2006-2010	42	23	27	S	NT	S
Portugal [33]	1999-2002	252	9.9	10.7	S	75.4	S
Portugal [34]	2005-2012	953	6 to 18	14 to 23	S	NT	S
Romania [72]	2009	100	17	18	S	91	NT
Spain [19]	1992-2001	1462	0.8 to 12.8	2.5 to 18.02	NT	85.2	NT
Spain [56]	1992-2009	212	NT	14.2	S	89.2	NT
Sweden [39]	2005	48	4	3.8	S	NT	NT
Turkey [16]	2000	33	9.1	21.2	S	NT	NT
Turkey [32]	2000-2001	54	9	7	S	91	S
Turkey [17]	2002-2003	300	20	20	S	NT	S
Turkey [46]	2002-2003	150	2.7	13.5	S	NT	S

Of the 30 studies, 25 showed that the isolates were consistently, over time and in different geographic areas, susceptible to penicillin. Nevertheless, antibiotic prophylaxis is not widely recommended in Europe due to an increase of resistance and allergies to penicillin [84]. In addition, evidences suggest that the use of penicillin may cause respiratory distress in the newborn [84]. GBS is greatly resistant to tetracycline and susceptible to vancomycin.

Resistance to macrolide antibiotics has been increasing in Europe and in the USA [7]. In this country, the resistance values to erythromycin vary from 7 to 21 % and to clindamycin from 3 to 15 % [2]. Based on the literature analyzed the resistance to erythromycin and clindamycin stood out. In France [18], *S. agalactiae* showed high resistance values for these antibiotics, 40 % for erythromycin and 37.8 % for clindamycin. It was also observed an increase in these resistances in GBS from Spain [19] and Portugal [34] during the periods studied. In general, the resistance values to erythromycin vary from 2.5 to 40 % and to clindamycin from 0.8 to 37.8 %. These antibiotics are recommended as alternatives for penicillin–allergic pregnant women. However, a resistance increase to these antibiotics has raised concerns on the prophylaxis and treatment of patients [2].

For susceptibility testing of GBS, disk diffusion or broth microdilution are the two methods recommended by the Clinical and Laboratory Standards Institute (CLSI) [11]. For the assessment of antimicrobial susceptibilities this guideline appears to be the most requested but others such as, European Committee on Antimicrobial Susceptibility Testing (EUCAST), Swedish Reference Group for Antibiotics (SRGA) and British Society for Antimicrobial Chemotherapy (BSAC) were also reported. The majority of the studies have used one of these recommended methods. In addiction E-test appears as a useful technique to identify the minimum inhibitory concentrations (MICs).

Conclusion

This review shows that there is a reasonable amount of European studies, covering 20 countries, containing information on the GBS carriers, on the prevalent serotypes and on the antibiotic resistances. Moreover, with the literature available it is evident that GBS is present in high rates, continuing to be a major public health problem. Due to the absence of universal guidelines and errors coming from incorrect screenings, the rates presented may be even higher and efforts should be made to improve epidemiological information. The diverse epidemiologic data between

countries should be analyzed to allow that strategies such as vaccination might be implemented.

Further research and consciousness of this problem are needed to develop universal guidelines for microbiological, screening and typing procedures for GBS diagnosis as well as updates on the intrapartum antibiotic prophylaxis, in order to decrease the burden of these infections.

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Supporting information

Table 3.3. – Characteristics of the GBS studies in European countries.

Author	Country	Study interval	Specimen collection site	Specimen collection time	Sample size	Culture methods	GBS identification methods	Guidelines mentioned
Areal et al. [15]	Portugal	2005	Rectovaginal	35-37 weeks' gestation	1523	No	No	CDC
Arisoy et al. [16]	Turkey	2000	Distal third of the vagina and the rectum (different swabs)	Not stated	310	Yes	Yes	NCCLS[a], CDC
Barbaros et al. [17]	Turkey	2002-2003	Cervix, lower one-third of the vagina and the rectum of pregnant women.	Not stated	300	Yes	Yes	CDC, NCCLS
Bergal et al. [18]	France	2011-2014	Vagina, urine and blood	Not stated	93	Yes	Yes	CDC, CA-SFM[b]
Betriu et al. [19]	Spain	1992-2001	Skin and soft tissues, urine, genital tract, respiratory tract, blood and others	Not stated	1462	No	Yes	NCCLS
Bianco et al. [20]	Italy	2014	Vaginal and rectal swab or both (different swabs)	Not stated	3988	No	No	CDC
Bjarnadóttir et al. [21]	Iceland	1994-1997	Vagina and rectal (different swabs)	23-36 weeks' gestation and at delivery	280	Yes	Yes	No

[a] NCCLS, National Committee for Clinical Laboratory Standards
[b] CA-SFM, French Society for Microbiology standards

Table 3.3. – Characteristics of the GBS studies in European countries (continue).

Author	Country	Study interval	Specimen collection site	Specimen collection time	Sample size	Culture methods	GBS identification methods	Guidelines mentioned
Brigtsen et al. [22]	Norway	2009- 2011	Rectovaginal	Not stated	1739	Yes	Yes	CDC,NWGA[c], EUCAST[d]
Brimil et al. [23]	Germany	2001-2003	Vaginal and rectal (different swabs)	Not stated	210	Yes	Yes	CDC
Brzychzy et al. [24]	Poland	2006-2010	Blood, oral cavity and external ear (newborns aged less than 7 days; neonates with early-onset GBS disease and from colonized neonates without any signs or symptoms of infection)	-	42	Yes	Yes	EUCAST, CDC
Busetti et al. [25]	Italy	2002-2005	Lower vaginal and rectal (different swabs)	35-37 weeks' gestation	5020	Yes	Yes	CDC
Decoster et al. [26]	Belgium	2001-2002	Rectovaginal	35-37 weeks' gestation	262	No	Yes	NCCLS
Domelier et al. [27]	France	2003	Vagina, gastric fluid of neonates without infection, cerebrospinal fluid (CFS), blood	35-37 weeks' gestation	711	No	No	ANAES[e], CA-SFM
Dore et al. [28]	Ireland	1997-1999	Blood, high vaginal swabs, umbilical cord swabs, placenta swabs and breast milk (from infants and women; asymptomatic patients)	Not stated	159	Yes	Yes	CDC
Ekelund and Konradsen [29]	Denmark	1984-2002	Blood and CFS	-	472	No	No	CDC, NCCLS

[c] NWGA, Norwegian Working Group on Antibiotics
[d] EUCAST, European Committee on Antimicrobial Susceptibility Testing
[e] ANAES, Agence nationale d'Accréditation en Santé

Table 3.3. – Characteristics of the GBS studies in European countries (continue).

Author	Country	Study interval	Specimen collection site	Specimen collection time	Sample size	Culture methods	GBS identification methods	Guidelines mentioned
El Aila et al. [30]	Belgium	2009-2010	Vaginal and rectal swab or both (different swabs)	35-37 weeks' gestation	300	Yes	Yes	CDC
El Aila et al. [31]	Belgium	2007	Vagina and rectum (different swabs)	35-37 weeks' gestation	150	Yes	Yes	NCCLS
Eren et al. [32]	Turkey	2000-2001	Vagina and rectum (different swabs). Umbilical and throat swabs	Not stated	54	Yes	Yes	CDC, NCCLS
Figueira-Coelho et al. [33]	Portugal	1999-2002	Vaginal isolates, urine, skin and soft tissue infections, and blood	Not stated	252	No	Yes	NCCLS
Florindo et al. [34]	Portugal	2005-2012	Rectovaginal	Not stated	953	No	Yes	CDC, NCCLS
Fluegge et al. [35]	Germany	2001-2003	Invasive isolates	-	296	No	No	NCCLS, EUCAST, DIN[f]
Gherardi et al. [36]	Italy	2002-2005	Invasive, noninvasive and colonizing isolates	Not stated	91	Yes	Yes	No
Gosiewski et al. [37]	Poland	2007-2009	Not stated	Not stated	75	No	No	No
Gudjónsdóttir et al. [38]	Sweden	2004-2009	Invasive isolates	Not stated	515	No	Yes	No
Hakansson et al. [39]	Sweden	2005	Lower vagina and rectum (different swabs)	Not stated	48	Yes	Yes	CDC, SRGA[g]
Hansen et al. [40]	Denmark	1999-2001	Vagina and rectum (different swabs)	48 h after birth	77	Yes	Yes	CDC
Imperi et al. [41]	Italy	2005-2008	Blood and/or CFS of infected newborns and infants	-	87	Yes	Yes	CDC

[f] DIN, Deutsche Insdustrie Norm
[g] SRGA, Swedish Reference Group for Antibiotics

Table 3.3. – Characteristics of the GBS studies in European countries (continue).

Author	Country	Study interval	Specimen collection site	Specimen collection time	Sample size	Culture methods	GBS identification methods	Guidelines mentioned
Janek et al. [42]	Slovakia	2000-2003	Vagina and rectum (different swabs)	35-36 weeks' gestation	754	Not available	Not available	Not available
Jaureguy et al. [43]	France	1997-1999	Vagina and rectum (different swabs)	35-37 weeks' gestation	370	Yes	Yes	CDC, ANAES
Jones et al. [44]	UK	2001-2003	Rectovaginal	34 weeks to full term	748	Yes	Yes	CDC
Joubrel et al. [45]	France	2007-2012	Invasive isolates	-	192	No	Yes	EUCAST, ANAES
Kadanali et al. [46]	Turkey	2002-2003	Vagina and rectum (different swabs)	Not stated	150	Yes	Yes	CDC, NCCLS
Kiely et al. [47]	Ireland	2004-2006	Vagina	Females aged between 15 and 54 years	2000	Yes	Yes	No
Kieran et al. [48]	Ireland	Not stated	Lower vagina and perianal site (different swabs)	Last four weeks of gestation	504	Yes	Yes	No
Kowalska et al. [49]	Poland	2001-2002	Cervix, vagina and perianal site	Not available	1678	Not available	Not available	Not available
Kunze et al. [50]	Germany	2004	Rectovaginal	35-37 weeks' gestation	869	Yes	Yes	CDC, NCCLS

Table 3.3. – Characteristics of the GBS studies in European countries (continue).

Author	Country	Study interval	Specimen collection site	Specimen collection time	Sample size	Culture methods	GBS identification methods	Guidelines mentioned
Lamagni et al. [51]	England and Wales	1991-2010	Invasive isolates	-	21386	No	No	BSAC[h]
Lamy et al. [52]	France	1990-2005	Human GBS strains	-	156	No	Yes	ANAES
Lito et al. [53]	Portugal	2004-2006; 2006-2007; 2009	Rectovaginal	Not stated	2639	No	No	No
Luan et al. [54]	Sweden	1988-1997	Invasive isolates	-	158	No	Yes	No
Martins et al. [55]	Portugal	2002-2004	Rectovaginal	Last trimester of pregnancy	269	No	Yes	No
Martins et al. [56]	Spain	1992-2009	Invasive isolates	-	212	No	No	NCCLS
Meehran et al. [57]	Ireland	2007-2011	Invasive and noninvasive isolates	Not stated	177	Yes	No	NCCLS
Melin et al. [58]	Belgium	2001-2003	Pregnant women's vagina, neonates and Adults	Not stated	187	No	No	No
Motlová et al. [59]	Czech Republic	2001-2002	Vagina and rectum (different swabs)	Women at childbirth	586	Yes	No	CDC, NCCLS
Óladóttir et al. [60]	Iceland	1975-2006	Blood or CFS cultures (newborn <90 days old))	-	89	Yes	No	No
Perez-Ruiz et al. [61]	Spain	2001-2002	Rectovaginal	35-37 weeks' gestation	30	Yes	No	No
Persson et al. [62]	Sweden	1998-2001	Blood, CFS and synovial fluid	-	161	No	Yes	No

[h] BSAC, British Society for Antimicrobial Chemotherapy

Table 3.3. – Characteristics of the GBS studies in European countries (continue).

Author	Country	Study interval	Specimen collection site	Specimen collection time	Sample size	Culture methods	GBS identification methods	Guidelines mentioned
Poulain et al. [63]	France	1994-1995	Vagina and perianal (different swabs)	After 28 weeks of pregnancy	2454	No	No	No
Poyart et al. [64]	France	2006-2007	Invasive isolates	-	109	No	Yes	NCCLS
Savoia et al. [65]	Italy	2005-2006	Vagina and rectum (different swabs)	35-37 weeks' gestation	73	Yes	Yes	CDC, NCCLS
Schoening et al. [66]	Germany	2001	Vaginal or rectal exudates of pregnant women, the ear, nose or gastric fluid of neonates, urine, ejaculate or urethral samples, skin and respiratory specimens	Not stated	338	No	No	NCCLS, DIN
Sensini et al. [67]	Italy	1993-1995	Lower vagina	At the time of delivery	2300	Yes	Yes	No
Skret-Magierlo et al. [68]	Poland and Ukraine	2010-2011	Lower vagina	At patient admission	100	No	No	No
Strakova et al. [69]	Czech Republic	2001-2002	Invasive and carrier GBS isolates from newborns	-	285	Not available	Not available	Not available
Trijbels-Smeulders et al. [70]	Netherlands	1997-1999	Blood, CFS and both	-	198	No	Yes	NCCLS
Tsolia et al. [71]	Greece	2000-2001	Vagina and rectum (different swabs)	≥ 35 weeks of gestation	1014	Yes	Yes	NCCLS
Usein et al. [72]	Romania	2009	Vagina	Not stated	100	No	Yes	NCCLS

Table 3.3. – Characteristics of the GBS studies in European countries (continue).

Author	Country	Study interval	Specimen collection site	Specimen collection time	Sample size	Culture methods	GBS identification methods	Guidelines mentioned
Valkenburg-van den Berg et al. [73]	Netherlands	2000-2002	Rectovaginal	35-37 weeks' gestation	1702	Yes	Yes	No
van der Mee-Marquet et al. [74]	Netherlands	Not stated	Vagina	35–38 weeks before delivery	500	Yes	Yes	ANAES
van Elzakker et al. [75]	France	1995-2004	Ante-partum rectovaginal cultures, blood, CFS and from both blood and CSF	Not stated	338	Yes	Yes	No
Volumenie et al. [76]	France	1994-1996	Vagina	35-37 weeks' gestation	3906	Yes	Yes	CDC
Weisner et al. [77]	England and Wales	2000-2001	Blood, CSF and joint aspirates obtained from infants aged ≤ 90 days	-	486	No	No	No
Whitney et al. [78]	Ireland	1999-2001	Lower vagina wall and cervix	20 and 32 weeks' gestation	1308	Yes	Yes	CDC
Yucesoy et al. [79]	Turkey	2001-2002	Anorectum, cervical, posterior fornix and vaginal speculum	35-37 weeks' gestation	1100	Yes	Yes	No

Table 3.4. – Percentage of GBS serotypes in different European countries.

Country	Study interval	Ia	Ib	II	III	IV	V	VI-IX	NT[a]
Belgium [31]	2007	10.4	10.4	10.4	20.8	12.5	20.8	NF[b]	14.5
Czech Republic [59]	2001-2002	22	9	12	33.2	0	13.9	3	7
Czech Republic [69]	2001-2002	13	8	14	42	3	13	1	6
Denmark [29]	1984-2002	17	7	6	59	1	4	NF	6
England and Wales [51]	1991-2010	24	9	10	40	1.7	14	NF	NF
England and Wales [77]	2000-2001	27	6	4	48	1	10	NF	3
UK [44]	2001-2003	25.8	15.7	9.4	26.4	0.63	18.9	1.1	2
France [52]	1990-2005	10.9	7	6.41	64.1	0.64	6.41	NF	4.49
France [75]	1995-2004	25.6	7.7	7.7	41	NF	17.9	NF	NF
France [27]	2003	7	12	5	22	19	22	NF	15
France [64]	2006-2007	28.2	5.1	2.5	61.5	NF	2.5	NF	NF
France [45]	2007-2012	23	4	6	57	2	7	NF	NF
France [18]	2011-2014	8	NF	19	19	1.3	44.6	2.6	5.4
Germany [23]	2001-2003	17	15	21	28	3	16	NF	NF
Germany [35]	2001-2003	15	5	5	65	1	8	NF	1
Greece [71]	2000-2001	19.4	12	26.9	22.4	3	9	7.5	NF
Iceland [60]	1975-2006	21	13	7	44	3	11	NF	NF
Ireland [48]	Not stated	23	7	17	30	1	13.2	NF	9
Ireland [28]	1997-1999	19.5	18.9	10.7	29.5	1.9	15.1	NF	4.4
Ireland [78]	1999-2001	18.6	4.7	14.8	19.4	NF	28	7	7.8
Ireland [57]	2007-2011	21.5	6.8	12.4	35	5.1	16.9	2.3	NF
Italy [67]	1993-1995	16.2	27	21.6	21.6	2.7	5.4	5.4	NF
Italy [36]	2002-2005	17.6	5.5	14.3	23.1	3.3	15.4	4.4	16.5
Italy [41]	2005-2008	9.3	5.3	6.7	72	1.3	5.3	NF	NF
Italy [65]	2005-2006	20.5	6.8	5.5	32.9	8.2	26.1	NF	NF
Netherlands [74]	Not stated	26	8	8	41	NF	18	NF	NF
Netherlands [70]	1997-1999	19	6	8	55	NF	7	NF	7
Norway [22]	2009-2011	15.7	9.9	14	24.9	14.5	16.9	4	1
Poland [24]	2006-2010	9	NF	27	50	NF	14	NF	NF
Poland [37]	2007-2009	20	8	15	35	5	17	NF	NF

[a] NT, Non-typeable
[b] NF, Not found.

Table 3.4. – Percentage of GBS serotypes in different European countries (continue).

Country	Study interval	Ia	Ib	II	III	IV	V	VI-IX	NT
Portugal [56]	1992-2009	22.2	6.1	5.2	55.7	1.4	6.6	NF	2.8
Portugal [33]	1999-2002	17.9	3.6	16.3	24.6	2	23.4	1.6	10.7
Portugal [55]	2002-2004	15.6	5.2	17.1	21.9	2.2	21.9	1.9	14
Portugal [34]	2005-2012	21	8	14	23	9	20	NF	4
Romania [72]	2009	19	3	26	26	9	17	NF	NF
Spain [61]	2001-2002	29	12.9	12.9	38.7	NF	6.5	NF	NF
Sweden [54]	1988-1997	16.5	8.9	9.5	53.2	1.9	10.1	NF	NF
Sweden [62]	1998-2001	9.3	7.5	5	36	5.6	36	NF	0.6
Sweden [38]	2004-2009	18	6	6	48	2	16	3	NF
Sweden [39]	2005	11	13	16	24	15	19	0.5	0.8
Turkey [32]	2000-2001	26	4	30	19	2	NF	NF	20

Chapter 4

Incidence and serotype characterization of *Streptococcus agalactiae* in a Portuguese hospital

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The results obtained in this chapter were presented to elements belonging to the Department of Neonatology and Clinical Pathology of Hospital Pedro Hispano.

Incidence and serotype characterization of *Streptococcus agalactiae* in a Portuguese hospital

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Abstract

Aims

Streptococcus agalactiae, commonly known as group B *Streptococcus* (GBS), has been recognized as a worldwide causative pathogenic agent of neonatal sepsis, meningitis and pneumonia. To better understand the behavior of *S. agalactiae* in pregnant women from a hospital from the North of Portugal, retrospective analyses were performed to describe epidemiological, clinical and microbiological characteristics of the isolates obtained.

Methods

Based on laboratorial records and the hospital's patient files, a 6-year retrospective study was performed to analyze *S. agalactiae* isolates from screened pregnant women between 35 and 37 weeks of gestation and hospitalized neonates from pregnant women between 24 and 41 weeks of gestation admitted in Hospital Pedro Hispano. Serotype characterization was also performed in 67 GBS strains.

Results

In 6692 pregnant women between 35 and 37 weeks of gestation screened between 2011 and 2016, a total of 1377 *S. agalactiae* isolates (21 %) were found. A high percentage (40 %) of unknown colonization status among hospitalized neonates from pregnant women between 24 and 41 weeks of gestations was also found. The

incidence of neonatal sepsis was 8.7 (95 % CI 7.0 to 10.8) cases per 1000 live births. Regarding serotype characterization, serotype III (22.4 %) was the most frequent, followed by serotype Ia (19.4 %) and serotypes Ib and V (both with 17.9 %).

Conclusion

High epidemiological values of GBS colonization and incidence were found in this study. In Portugal studies on the epidemiology and behavior of *S. agalactiae* remain limited, reinforcing the importance and need for *S. agalactiae* screening across the country.

Keywords

Streptococcus agalactiae, Urogenital colonization, Pregnancy, Perinatal pathology, Epidemiology.

Introduction

Streptococcus agalactiae, also known as group B *Streptococcus* (GBS), is a commensal that colonizes the gastrointestinal and genitourinary tracts of healthy adults, particularly in women [1,2]. GBS members are distinguished by their type-specific capsular polysaccharides (CPS), which are essential for GBS virulence. Currently, they can be differentiated into 10 immunologically unique CPS (Ia, Ib, and II to IX), and their distribution varies worldwide according to geographical location, time period of study and ethnicity [1,3,4].

In the 1970s, GBS was recognized as the main cause of early-onset disease (within the first 6 days of life) and late-onset disease (between 1 week and 3 months of age) in newborns, with mortality rates up to 10 % [5,6]. GBS can be acquired through vertical transmission by direct contact with the bacterium in the uterus or through vaginal secretions from the colonized birth canal during labor [7]. Maternal and neonatal GBS colonization rates have been found to vary between different countries, different ethnic groups, gestational age and laboratory procedures on the sampling [2,8].

The Centers for Disease Control and Prevention (CDC) 2010 Guidelines for Prevention of Perinatal Group B Streptococcal Disease recommend screening women between 35 and 37 weeks of pregnancy to determine GBS carrier status and prophylactic antibiotic treatment during labor for women at risk of transmitting this pathogen [9]. The recommended screening and prophylaxis strategies by CDC have been very successful, reducing the incidence of early-onset GBS by more than 80 % [10].

Clinical trials demonstrated the efficiency of both penicillin and ampicillin as intravenously administered intrapartum agents for the prevention of *S. agalactiae* infections. As GBS is susceptible to penicillin, this is the first drug of choice for the prophylaxis and treatment [11] However, macrolides or lincosamides are used as alternative drugs in patients with a history of beta-lactams allergy [12].

Maternal vaccination against GBS aimed to reduce maternal colonization and to enhance transplacental transfer of anti-GBS antibody to the fetus [13]. Serotypes Ia, Ib, II and III have shown to be predominant in many parts of the world [14,15]. As serotype distribution varies worldwide, it is important to identify their distribution and possible changes in different populations [16].

Recent analyses based on published studies have reported a mean global incidence of GBS of 0.53 cases per 1000 live births (LB) [17]. Overall, the incidence of GBS infection ranges from 0.17 to 3.06 cases per 1000 LB in low-income and middle-income countries [18] and the mortality rate is higher than in developed countries (10 %–60 % compared with 7%–11%) [19]. In Europe the incidence is 0.5–2 cases per 1000 LB, with a mortality rate of 4 %–10 % [3].

To better understand the behavior of *S. agalactiae* in this study, retrospective analyses were performed to describe epidemiological, clinical and microbiological characteristics of the isolates obtained from screened pregnant women between 35 and 37 weeks of gestation and hospitalized neonates from pregnant women between 24 and 41 weeks of gestation in Hospital Pedro Hispano, during a 6-year period (2011–2016). Furthermore, GBS serotype characterization of 67 GBS isolates from pregnant women was performed.

Materials and methods

Retrospective study

Patients

This retrospective study is based on clinical and microbiological records from 6687 screened pregnant women between 35 and 37 weeks of gestation and 1277 hospitalized neonates from pregnant women between 24 and 41 weeks of gestation admitted in Hospital Pedro Hispano. The study was conducted between 2011 and 2016 with a total of 9223 deliveries. Microbiological information on the GBS colonization status of screened pregnant women was collected from the clinical pathology laboratory records (database Clinidata), and clinical data about GBS colonization, prophylaxis and sepsis outcome of hospitalized neonates were obtained from the hospital's patient files.

Serotype characterization

Sampling and growth of *S. agalactiae*

Serotype characterization was performed in 67 GBS strains isolated between January 2017 and May 2017. The samples were collected with a swab from the vagina and rectum (two swabs), which were introduced in Todd-Hewitt (TH) broth for enrichment, followed by subculture onto commercial chromogenic agar, Granada agar (BioMérieux), and grown for 14–48 hours under anaerobic conditions at 35 °C ± 2 °C, as recommended by the CDC guidelines (internal procedure n.2637.0). The different isolates were stored at −80 °C.

Cultures for serotype analysis were then cultivated on Granada agar for 48 hours at 36 °C under anaerobic conditions.

DNA extraction and multiplex PCR assay

For DNA extraction, single GBS colonies were suspended in 100 µL of Tris-EDTA buffer, and further extraction was performed according to the manufacturer's instructions with the EZNA Bacterial DNA Kit.

Serotype characterization of *S. agalactiae* was done though a multiplex PCR method for serotypes Ia, Ib and II to IX using the primers described by Poyart et al. [20] and Imperi et al. [21] (Table 4.4. – Supporting Information). Non-typeable isolates were designated as NT.

This assay was performed in a final volume of 25 µL containing 1x NZYTaq 2x Green Master Mix, 0.2 µM of each primer and 2 µL of the extracted DNA. Two primer mixtures (mixture 1: Ia–IV; and mixture 2: V–IX) were used in separate PCRs. The samples were amplified in a MyCycler Thermal Cycler under the following conditions: 95 °C—5 min, 15 × (95 °C—1 min, 54 °C—1 min, 72 °C—2 min), 25 × (95 °C—1 min, 56 °C—1 min, 72 °C—2 min), 72 °C—10 min. The PCR products were separated in 1 % agarose gel stained with Green Safe Premium. Gel visualization and documentation were conducted in a GelDoc and GenoPlex system.

Statistics

GBS incidence rates were expressed as number of cases per 1000 live births $\left\{i = \frac{number\ of\ cases\ admitted\ in\ the\ 6\ years}{total\ number\ of\ live\ births\ in\ the\ 6\ years} \times\ 1\,000\right\}$ and the Wilson score interval method was used to calculate all 95 % CIs [22]. Serotype distribution was described as a frequency (number and percentage of strains).

A $\chi 2$ analysis of both the colonization status and the prophylaxis against the neonatal infection was performed at a significance level of 0.05. The colonization status

was considered as positive, negative or unknown. Prophylaxis was considered complete when given two or more doses, incomplete when less than two doses and none when no antibiotic was given. The antibiotics used were intravenous penicillin G with an initial dose of 5 million units followed by 2.5 million units 4/4 hours until birth, or intravenous ampicillin with an initial dose of 2 g followed by 1 g 4/4 hours until birth; if the pregnant patient was allergic to penicillin, intravenous erythromycin and clindamycin 900 mg 8/8 hours until birth.

Results and discussion

Retrospective study

In this study, 1376 (21 %) of the 6687 screened pregnant women between 35 and 37 weeks of gestation (Figure 4.1.A) during the 6-year study were found to be positive for *S. agalactiae* and were all new cases. This prevalence is consistent with international values of GBS colonization of healthy adults, which varies between 10 % and 30 % [8,23]. Several other Portuguese studies assessed maternal colonization rates. In the North of Portugal, four studies [24–27] have documented rates of 17 %, 18 %, 19.7 % and 34.9 %. In the South of Portugal, rates of 13.9 % and 21.1 % have been found by Lito et al. and Rodrigues [28,29]. These differences can be due to multiple factors such as geographical location, ethnicity, GBS detection methods, gestational age and differences in study setting (hospital, regional and national) [2,8,30]. Variations on the maternal GBS carriage rates have been found to vary depending on the race and ethnic groups within the same country as demonstrated in a US study by Regan et al. [31] with colonization rates of 21.2 % for black, 20.9 % for hispanic and 13.7 % for white women.

For the isolation of *S. agalactiae*, the CDC guidelines recommend the enrichment in selective broth media such as TH broth supplemented with gentamicin (8 µg/mL) and nalidixic acid (15 µg/mL) or with colistin (10 µg/mL) and nalidixic acid (15 µg/mL), followed by subculture onto tryptic soy agar with 5 % defibrinated sheep blood, Columbia agar with colistin and nalidixic acid, or a commercial chromogenic agar [9]. However, not all laboratories that perform GBS screening follow these recommendations, using other media that may allow the growth of the entire vaginal and rectal microbiota. In these cases, GBS presence can be camouflaged leading to the obtainment of false-negative results. Moreover, GBS can be acquired during the period between screening and delivery, and due to antibiotics and feminine hygiene products, which reduce the sensitivity of the cultural method [32–34] false GBS negative results may occur [9].

Molecular biology-based assays have been shown to be consistent and more rapid than the standard culture-based method. Therefore, antigen detection and phylogenetic GBS characterization by PCR, directly from combined vaginal and rectal swab specimens, have been evaluated in terms of sensitivity and specificity [32,35]. Moreover, the colonization rate obtained by cultural methods is lower than by molecular methods [33,36,37]. PCR is a technique that allows to confirm that the majority of the negative results are truly negative and that the positive ones should be confirmed by other methods such as culture or serology [32]. Cultures would still be necessary to provide antimicrobial susceptibility information, especially in patients with penicillin allergy [33,38].

The studied population (n=6687) was aged between 14 and 46 years old. Although no statistical significance (P=0.214, P>0.05) was observed, a gradual and slight increase of GBS colonization occurred with maternal age (Figure 4.1.B). Joachim et al. [39] also showed that GBS is isolated more frequently from women of the age group 30–34 (32.1 %) compared with women aged <20 years (15.4 %), with no statistical significance. These results highlight that GBS colonization can be influenced by multiple factors.

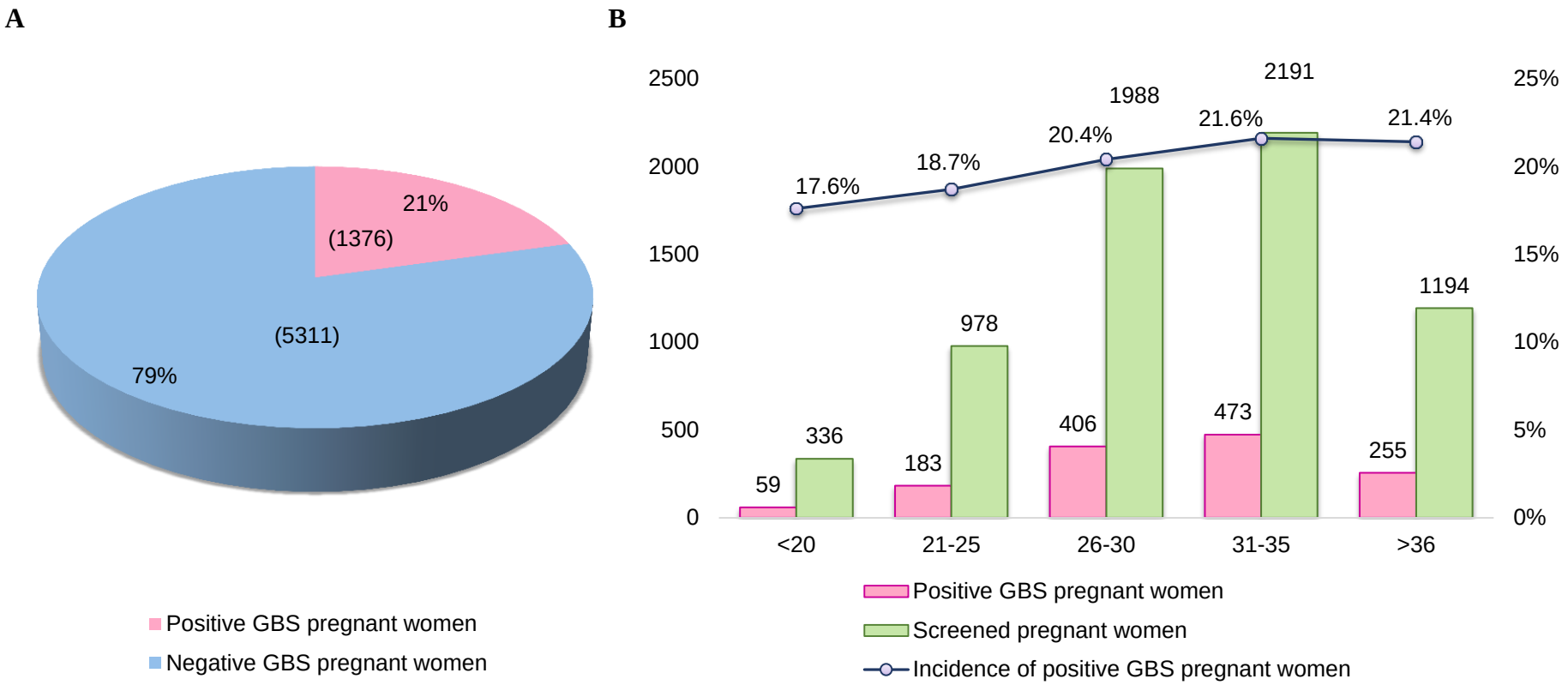

Figure 4.1. – (A) Incidence of positive and negative group B *Streptococcus* (GBS) colonization on the 6687 screened pregnant women and (B) distribution by maternal age of positive GBS screened pregnant women.

Based on clinical information of the 1277 hospitalized neonates, positive, negative and unknown GBS colonization values of pregnant women between 24 and 41 weeks of gestation are shown in figure 4.2.A. Of these, 173 (14 %) were positive for GBS, 595 (46 %) were negative and 509 (40 %) had an unknown colonization status. The high average of 40 % of unknown GBS colonization was discriminated by gestational age (Figure 4.2.B), and the results showed that unknown colonization status is more frequent in gestational ages of 34 (17.7 %), 33 (11.8 %), 32 (11.6 %) and 35 (10.6 %) weeks. Women at term with unknown GBS colonization status normally do not perform screening cultures as it takes up to 48 hours to arrive to a final result. Also, pregnancies with lower gestational ages and elective caesarean sections sometimes have no opportunity to be screened. Therefore, CDC recommended that these women should be managed according to the presence of intrapartum risk factors [9].

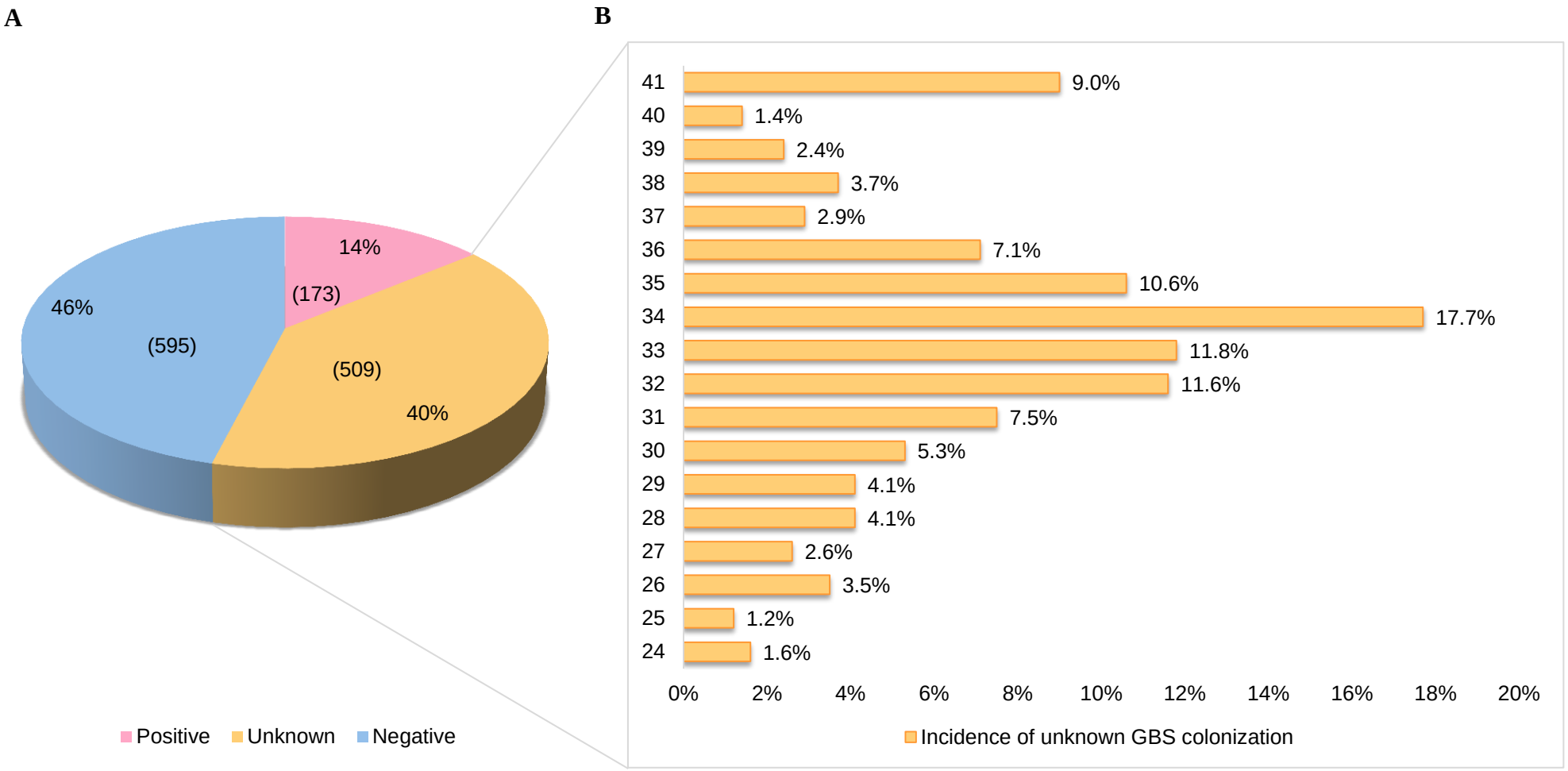

Figure 4.2. – (A) Incidence of positive, negative and unknown group B *Streptococcus* (GBS) colonization of pregnant women (24–41 weeks of gestation) with neonates who were hospitalized and (B) distribution (percentage) by gestational age of pregnant women with unknown colonization status.

Considering a total of 9223 deliveries that occurred in the 6 years, the incidence of neonatal sepsis was 8.7 cases per 1000 LB (95 % CI 7.0 to 10.8). The high values of sepsis from negative GBS women that must be due to other microorganisms' infections are not further addressed because they are out of the scope of this study. The Portuguese Pediatric Surveillance Unit-Portuguese Society of Pediatrics observed, between 2001 and 2005, a GBS incidence of 0.54 per 1000 LB in Portugal with a range between 0.1 and 0.9 cases per 1000 LB [40]. The value presented in our study is much higher than the indicated by these authors and by the one indicated for Europe, which is 0.5–2 cases per 1000 LB [3]. However, a more recent study also performed in the North of Portugal reported a similar incidence of 9 cases per 1000 LB [41]. In addition, Neto [40] demonstrated that even in a small country as Portugal, the incidence can vary according to geographical areas (North 0.9/1000 LB; Centre 0.4/1000 LB; Lisbon and Tagus Valley 0.4/1000 LB; Algarve 0.1/1000 LB; and Islands 0.2/1000 LB).

Regarding prophylaxis with intrapartum antibiotic administration, in 1023 (80.1 %) pregnant women prophylaxis was not initiated, in 139 (10.9 %) was performed completely and in 115 (9 %) the prophylaxis was incomplete (table 4.1.). Thus, the incidence of a neonate sepsis outcome in pregnant women that initiate prophylaxis, between 2011 and 2016, was 7.4 cases per 1000 LB (95 % CI 5.8 to 9.3). This value is lower when compared with the global incidence of neonate sepsis of 8.7 cases per 1000 LB (95 % CI 7.0 to 10.8) obtained in this study. This can be explained by the prophylaxis performed; however, it was not a significant decrease.

Significant statistical significances were found between maternal GBS colonization status and neonatal infection ($P<0.01$; $P\leq0.05$) and between prophylaxis performed and neonatal infection ($P<0.001$; $P\leq0.05$) (table 4.1.).

Table 4.1. – Neonatal sepsis outcome *versus* maternal colonization status and prophylaxis.

Sepsis	**Colonization status**			**Total**	**Prophylaxis**			**Total**
	Positive	Negative	Unknown		Complete	Incomplete	None	
Yes	80 (6.3 %)	229 (17.9 %)	170 (13.3 %)	479 (37.5 %)	68 (5.3 %)	61 (4.8 %)	348 (27.3 %)	477 (37.4 %)
No	93 (7.3 %)	366 (28.7 %)	339 (26.5 %)	798 (62.5 %)	71 (5.6 %)	54 (4.2 %)	675 (52.9 %)	800 (62.6 %)
Total	173 (13.5 %)	595 (46.6 %)	509 (39.9 %)	1277 (100 %)	139 (10.9 %)	115 (9 %)	1023 (80.1 %)	1277 (100 %)

Also, an association (P=0.135, P>0.05) between screened and non-screened pregnant women versus sepsis outcome was found (table 4.2.). Areal et al. [41] reported comparable significant results and defend that prophylaxis may reduce/eliminate perinatal infection.

Table 4.2. – Neonatal sepsis outcome *versus* screened and not screened pregnant women.

	Sepsis		**Total**
	Yes	No	
Screened	309 (24.2 %)	459 (35.9 %)	768 (60.1 %)
Not screened	170 (13.3 %)	339 (26.6 %)	509 (39.9 %)
Total	479 (37.5 %)	798 (62.5 %)	1277 (100 %)

Serotype characterization

Serotype characterization of *S. agalactiae* isolates was performed by a multiplex PCR method (figure 4.3.), and the results obtained confirmed their affiliation to GBS. Of the 67 collected GBS isolates, serotype III (22.4 %) was the most frequent, followed by serotype Ia (19.4 %) and serotypes Ib and V both with 17.9 % (table 4.3.). Serotypes VII, VIII and IX were not found and 3 % isolates were NT.

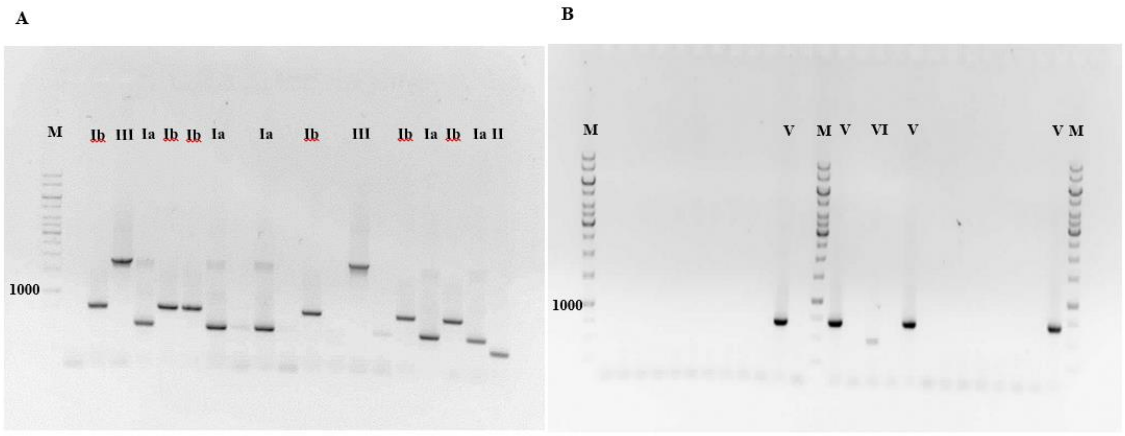

Figure 4.3. – **A** – Multiplex PCR for detection of capsular types Ia-IV and **B** – Multiplex PCR for detection of capsular types V-IX.

Table 4.3. – Distribution of capsular types among 67 *S. agalactiae* isolates.

	Ia	Ib	II	III	IV	V	VI	VII	VIII	IX	NT[a]	Total
n=	13	12	7	15	5	12	1	0	0	0	2	67
%	19.4	17.9	10.4	22.4	7.5	17.9	1.5	0	0	0	3	100

In Portugal, serotype distribution surveys have been performed and described in previous studies, all in the south of the country [11,42–44]. A laboratory-based surveillance program that involved 22 Portuguese medical centers (from north to south) [45] was also performed and has shown rates of 58.7 % for serotype III, 22.0 % for serotype Ia, 5.5 % for serotype Ib and 4.1 % for serotype V.

[a] NT – Non-typeable

The most common methods for serotyping GBS are latex agglutination and PCR-based methods for GBS capsular typing (targeting genes in the cps operon) [46,47]. Brigtsen et al. [46] found capsular genotyping methods more reliable (only 0.7 % isolates not identified) than typing by latex agglutination (13.8 % NT). Latex agglutination method may fail on the isolate typing due to lack of or low expression of CPS and due to the quality of the antibodies used [47].

Despite these findings and in contrast with our study, all the previous Portuguese studies presented have used latex agglutination for GBS serotyping except one. In a study between 2005 and 2007, Florindo et al. [43] has used a PCR-based method for capsule genotyping and has documented that the most frequent GBS serotypes were III (35 %), V (33 %), Ia (16 %) and II (10 %). However, this is from a different geographical region, Lisbon, in the South of Portugal, which may explain the differences encountered from ours results. Epidemiological distribution of these 10 serotypes can vary depending on geographical region, population under study and the source of the bacterial isolate [48]. Studies from Gambia [49] and Egypt [50] have reported serotype V as the most common serotype, while in South Africa and Morocco [13,51,52] serotypes Ia and III are dominant with a range of 30 %–40 % and serotype V only with 10 % of isolates.

Overall, in Europe including Portugal, a very conserved and consistent serotype distribution is observed where types Ia and III are dominant and types II and V have been frequently, but less, described too [11,51].

Conclusion

This study has revealed a high GBS colonization rate (21 %) on screened pregnant women, which led to a high probability of neonatal infections in the neonates (8.7 cases per 1000 LB). This fact shows a continuous need for colonization screenings and for a correct prophylaxis that can reduce/eliminate the direct contact, through vertical transmission, of neonate with *S. agalactiae*. It also suggests that the number of screenings should be increased at gestational ages between 24 and 41 weeks due to the high rate found of unknown GBS colonization (40 %).

The data obtained strongly support the need for revision of the current protocol performed in the hospital and the consideration of other screening methodologies, such as bacterial identification by PCR directly from combined vaginal and rectal swab specimens. This technique allows more viable and faster results in pregnant women at term with unknown GBS colonization status, pregnancies with lower gestational ages and elective caesarean sections. Although PCR is a rapid method, it is not a

replacement for cultural methods because (1) positive results should always be confirmed and (2) it does not provide antimicrobial susceptibility.

Knowledge of local distribution of the different serotypes of *S. agalactiae* is important for the development of an effective vaccine. According to the results a conjugated vaccine containing antigenic profiles of the serotypes Ia, Ib, II, III, IV and V would cover approximately 95 % of the serotypes carried by pregnant women in our study. This innovative alternative could reduce and prevent GBS invasive infections through pregnant women immunization. There is a lack of information in the north of the country regarding GBS serotyping. Therefore, further studies across the country on this topic are needed, which should be combined with a correct methodology for clinical GBS isolation.

Acknowledgements

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Supporting Information

Table 4.4. – Primer identification and PCR products specific for the studied gene targets [20, 21].

Primer name	Sequence (5' to 3')	Gene target (s)	PCR products (bp)
Ia-F	GGTCAGACTGGATTAATGGTATGC	*cps*1aH	521 and 1826
Ia-R	GTAGAAATAGCCTATATACGTTGAATGC	*cps*1aH	
Ib-F	TAAACGAGAATGGAATATCACAAACC	*cps*1bJ	770
Ib-R	GAATTAACTTCAATCCCTAAACAATATCG	*cps*1bK	
II-F	GCTTCAGTAAGTATTGTAAGACGATAG	*cps*2K	397
II-R	TTCTCTAGGAAATCAAATAATTCTATAGGG	*cps*2K	
III-F	TCCGTACTACAACAGACTCATCC	*cps*1a/2/3I	1826
III-R	AGTAACCGTCCATACATTCTATAAGC	*cps*1a/2/3J	
IV-F	GGTGGTAATCCTAAGAGTGAACTGT	*cps*4N	578
IV-R	CCTCCCCAATTTCGTCCATAATGGT	*cps*4N	
V-F	GAGGCCAATCAGTTGCACGTAA	*cps*5O	701
V-R	AACCTTCTCCTTCACACTAATCCT	*cps*5O	
VI-F	GGACTTGAGATGGCAGAAGGTGAA	*cps*6I	487
VI-R	CTGTCGGACTATCCTGATGAATCTC	*cps*6I	
VII-F	CCTGGAGAGAACAATGTCCAGAT	*cps*7M	371
VII-R	GCTGGTCGTGATTTCTACACA	*cps*7M	
VIII-F	AGGTCAACCACTATATAGCGA	*cps*8J	282
VIII-R	TCTTCAAATTCCGCTGACTT	*cps*8J	
(IX) cpsI-7-9-F	CTGTAATTGGAGGAATGTGGATCG	*cps*I	229
(IX) cpsI-9-R	AATCATCTTCATAATTTATCTCCCATT	*cps*I	

Chapter 5

General discussion and concluding remarks

Chapter 5

General discussion and concluding remarks

As a leading cause of neonatal invasive disease, *Streptococcus agalactiae* has become an unquestionable important human pathogen. Given the clinical relevance of this microorganism it is essential to screen in pregnant women in order to decide whether or not to apply prophylaxis measures during childbirth.

In Portugal, there are only few studies about the epidemiological characterization of GBS isolates.

In the study performed between 2011 and 2016 in HPH, 21 % of the screened pregnant women between 35-37 weeks of gestation were GBS positive. Close percentages were also found in other two Portuguese studies [1,2]. (Figure 5.1.)

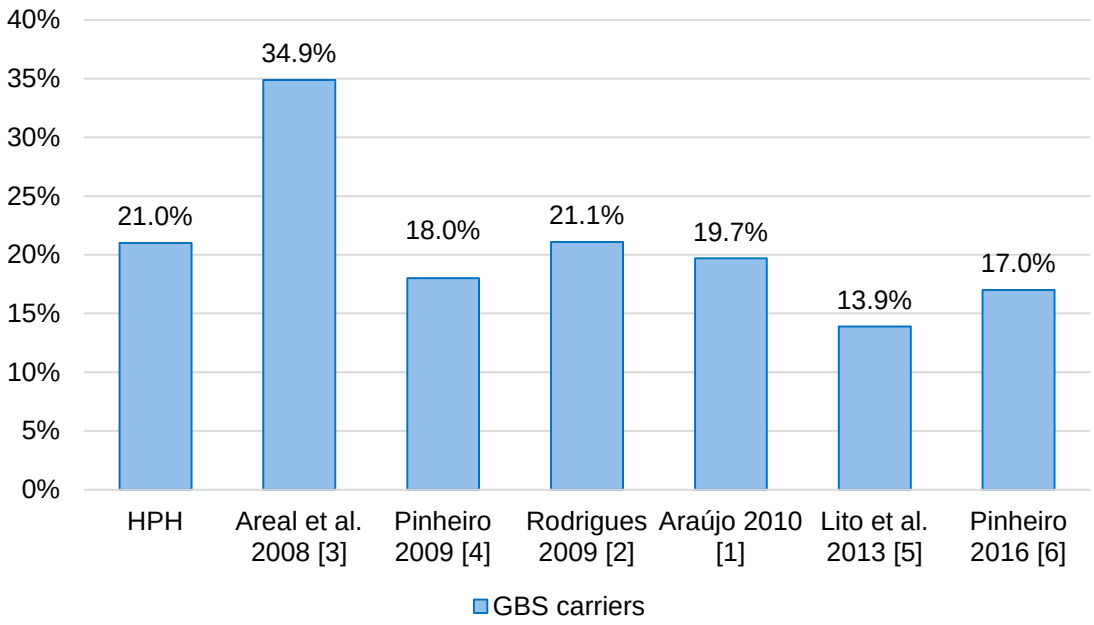

Figure 5.1. – Percentage of GBS carriers in different Portuguese studies. Adapted from Chapters 3 and 4.

The results observed in HPH are consistent with the values reported in Chapter 3 relative to Europe (6.5 % to 36 %). In addition, a clear geographic variation is observed among different Portuguese sampling locations (13.9 % to 34.9 %) as reported in other studies at European level. These variations can be due to the sampling procedures, to the different ethnic groups and gestational age in which screening is done [7].

As described in most European countries (Chapter 3), the prevalence of the GBS serotypes in Portugal also differ according to geography (Figure 5.2.).

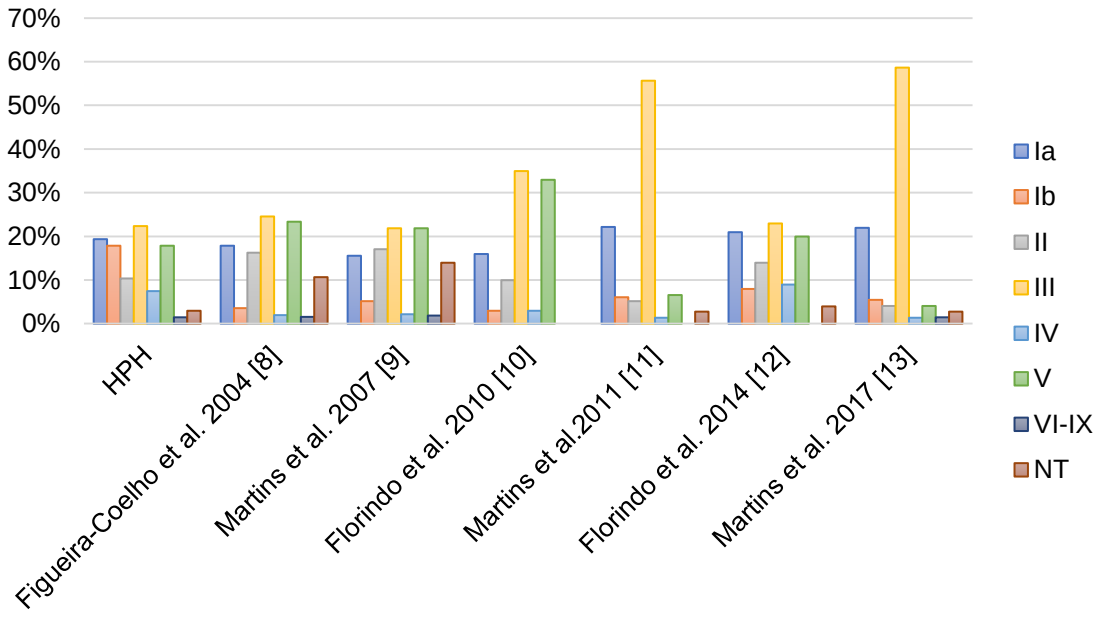

Figure 5.2. – Prevalence of GBS serotypes among different Portuguese studies. Adapted from Chapters 3 and 4.

The comparison of the prevalence of serotypes in different locations in Portugal revealed that capsular types III (34 %) and Ia (19 %) are the most frequent followed by serotypes V (18 %) and II (11 %). These serotype values are very similar to those found in Europe (III (37 %), Ia (18 %), V (15 %), and II (12 %)). Taken together, this reveals that a stable GBS population is responsible for the colonization in pregnant women. However, the variations presented in each country reflect a geographical specificity, which may be due to different genetic lineages responsible for GBS infections and the capacity to resist to GBS infection/ colonization by the different ethnic groups.

In this study, a percentage of 3 % of GBS isolates were non typeable, a very similar percentage to that reported by Florindo et al. (4 %) [12], Martins et al. (3 %) [11] and Martins et al. (3 %) [13]. Although the genetics of the non-typeable (NT) phenotype is not known [9] the absence of reaction with the available antisera (in latex agglutination test) may be explained by the deficit in the production of capsular polysaccharides, the variation in the expression of the genes coding for polysaccharide production [15], the existence of mutations or the insertion of mobile genetic elements in the cps locus of *S. agalactiae* of human or bovine origin [15,16].

Monitoring GBS infections is an important step to implement preventive strategies such as vaccine, which should be administered before pregnancy in adolescents and in women of childbearing age [17]. Considering the Portuguese studies included in this thesis, a vaccine against serotypes Ia, Ib, II, III and V could offer protection to 89 % of the population. An universal vaccine is the most promising approach against GBS infections and will overcome the limitations associated with intrapartum antibiotic prophylaxis. However, other possible prevalent serotypes in other parts of the world may be consider. For this, accurate studies on the GBS epidemiology, host specificity, virulence potential and antibiotic resistance phenotype are essential.

The implementation of PCR methodology on the current performed national protocol for GBS identification, screening and prophylaxis would improve the characterization of GBS populations and will provide better results in the control of GBS incidence. More screenings and the implementation of this technique applied to pregnant women at term with unknown GBS colonization status, pregnancies with lower gestational ages, elective cesarean sections and sampling blood of neonates will diminish the unknown GBS colonization percentage (40 %) and will provide more viable and faster results.

The two studies presented in this thesis allow an European and a National insight into the population of GBS. In general, they contribute to a better understanding of the epidemiology of GBS, highlighting the importance of universal protocols. These protocols will allow the identification of GBS colonized women even without obstetric risk factors permitting to achieve a better rationalization of the use of intrapartum antibiotics.

5.1. References

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