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ABTRACT

Introduction: *Streptococcus agalactiae* or group B streptococci (GBS) are the main etiologic agent of early neonatal sepsis in developed countries. This microorganism belongs to the gastrointestinal tract microbiota from where it can colonize the vagina and be vertically transmitted to child before or at birth and subsequently cause infection in the newborn. Approximately 50% of newborns from pregnant women harboring GBS become colonized, with 1-2% developing early neonatal infection if no preventive intervention is performed. **The aim of this study was to characterize and compare the serotype, virulence factors and antimicrobial resistance of GBS isolates from pregnant women and newborns from several hospitals in Catalonia.**

Methods: We analyzed 242 GBS strains including 95 colonizers, 68 pathogenic strains isolated from pregnant women and 79 strains isolated from neonates **with sepsis** to determine serotype, virulence and antimicrobial resistance.

Results: Distribution of the serotypes was different among the three groups, being serotypes Ia and II significantly more frequent among colonizing strains (p=0.001 and 0.012, respectively). The virulence factors *bca* and *scpB* were significantly more frequent among neonatal strains than pathogenic or colonizing strains (p=0.0001 and 0.002, respectively). Pathogenic strains were significantly more resistant to erythromycin, clindamycin and azithromycin than the non-pathogenic counterparts.

Conclusions: Taking into account that neonatal sepsis is an important problem worldwide, surveillance of the epidemiology, antimicrobial resistance and virulence of GBS **at local level** could provide great knowledge about these microorganisms and help

to improve treatment **and to prevent the neonatal invasive infection caused by this microorganism.**

RESUMEN

Introducción: *Streptococcus agalactiae* o estreptococos del grupo B (SGB) es el principal agente etiológico de la sepsis neonatal temprana en los países desarrollados. Este microorganismo pertenece a la microbiota del tracto gastrointestinal desde donde puede colonizar la vagina y ser transmitido verticalmente al niño antes o al nacer y posteriormente causar infección en el recién nacido. Aproximadamente el 50% de los recién nacidos de mujeres embarazadas que albergan GBS se colonizan, con 1-2% desarrollando infección neonatal temprana si no se realiza intervención preventiva. **El objetivo de este estudio fue caracterizar y comparar serotipos, factores de virulencia y la resistencia a los antimicrobianos de aislamientos de SGB de mujeres embarazadas y neonatos procedentes de varios hospitales de Cataluña.**

Métodos: Se analizaron 242 cepas de GBS incluyendo 95 colonizadoras, 68 cepas patógenas aisladas de mujeres embarazadas y 79 cepas aisladas de neonatos **con sepsis** para determinar serotipo, virulencia y resistencia antimicrobiana.

Resultados: La distribución de los serotipos fue diferente entre los tres grupos, siendo los serotipos Ia y II significativamente más frecuentes entre las cepas colonizadoras ($p = 0,001$ y $0,012$, respectivamente). Los factores de virulencia *bca* y *scpB* fueron significativamente más frecuentes entre las cepas neonatales que entre las patógenas o colonizadoras ($p = 0,0001$ y $0,002$, respectivamente). Las cepas patógenas fueron significativamente más resistentes a eritromicina, clindamicina y azitromicina que las no patógenas. **Conclusiones:** Teniendo en cuenta que la sepsis neonatal es un problema importante a nivel mundial, la vigilancia de la epidemiología, la resistencia a los

antimicrobianos y la virulencia del SGB **a nivel local** podría proporcionar un gran conocimiento de estos microorganismos y ayudar a mejorar el tratamiento **y la prevención de la infección invasiva causada por este microorganismo.**

Keywords: *Streptococcus agalactiae*, virulence, serotypes, antimicrobial resistance.

INTRODUCTION

Streptococcus agalactiae (group B streptococcus [GBS]) is an opportunistic pathogen that colonizes the gastrointestinal and genitourinary tract of healthy people and is responsible for severe diseases in susceptible hosts[1]. Moreover, this specie is a leading cause of invasive infection in newborns, causing early neonatal sepsis in developed countries. In Spain, it has been reported that 10-18.5% of pregnant women are colonized by GBS[2]. Colonization of the vagina by GBS in pregnant women is of great importance because it increases the risk of infection, due to it can be vertically transmitted to the child before or at birth, causing infection in the newborn. Infection by GBS may arise from a prematurely ruptured amniotic membrane which becomes infected or from infected amniotic fluid (chorioamnionitis) or preterm delivery in a mother colonized by these bacteria, presenting a much higher risk of infecting the offspring due to immaturity of the immune system[3]. Approximately 50% of infants from GBS-colonized women become colonized, with 1-2% of these newborns developing early or late neonatal infection if no preventive intervention is performed[4]. To avoid this enhanced risk of vertical transmission, the Center of Disease Control (CDC-1996) recommended the screening of all pregnant women at 35-37 weeks of gestation to determine possible GBS colonization[5]. Penicillin and ampicillin are the antibiotics of choice for treatment or intrapartum prophylaxis of GBS, followed by

first-generation cephalosporins. In the case of patients allergic to β-lactams, clindamycin, erythromycin and vancomycin are the antimicrobials of choice[6, 7].

When implemented, the use of these prophylactic measures results in a decrease in the incidence of infection by GBS[8]. In Spain, an example of the success of these prophylactic measures was demonstrated in a study carried out in 10 hospitals in Barcelona (Spain). It was found that the incidence of GBS as a cause of neonatal sepsis was reduced from 1.92/1,000 newborns in 1994 to 0.26/1,000 newborns in 2001 ($p < 0.001$) [2].

Ten GBS serotypes have been classified (Ia, Ib and II to IX), according to recognized capsular polysaccharides that are considered to be a major virulence factor in invasive disease caused by this microorganism[10]. Serotypes Ia, Ib, III and V are the most frequently involved in invasive infection[11]. Additionally, GBS can develop different pathogenic mechanisms that allow colonization and invasion in different niches within the host being able to avoid and suppress the host immune response.

The aim of this study was to characterize and compare the serotype, virulence factors and antimicrobial resistance of GBS **isolates** from pregnant women and newborns from several hospitals in Catalonia**.**

MATERIALS AND METHODS

Clinical sample and bacterial isolates.

Two hundred forty-two *S. agalactiae* **isolates** were collected **from 242 patients** from 5 Catalan hospitals (Hospital Clínic, Vall d'Hebron, San Joan de Déu, Parc Taulí and CatLab) from 2010 to 2016. Among these **isolates**, 95 were obtained from vaginal, rectal-vaginal and endocervical swabs from asymptomatic pregnant women at 35 to 37 weeks of gestation and categorized as colonizing; 68 were obtained from urine, blood, amniotic

fluid, and endometrial and placental samples from symptomatic women and categorized as pathogenic **isolates**; and 79 **isolates** were isolated from blood, cerebrospinal and amniotic fluid samples of newborns with neonatal sepsis. **Samples arriving to the different laboratories of Microbiology were cultured in Granada agar and selective Todd-Hewwitt broth specific for GBS isolation.** Suspected colonies were confirmed by MALDI-TOF.

Serotyping

Determination of the capsular type or serotype was carried out by a multiplex-PCR using a set of primers described previously[4].

Virulence profile

Different virulence determinants, including *bac* (encoding alpha subunit of the C protein), *bca* (encoding beta subunit of the C protein), ***cyl*(E)** (encoding cytolysin-haemolysin), *rib* (Alp family protein Rib), ***hyl*(B)** (encoding hyaluronidase protein), ***pep*(B)** (encoding oligopeptidase protein), ***scp*(B)** (encoding an invasin), ***fbs*(B)** (encoding fibrinogen-binding protein mediating invasivity), ***spb*(1)** (encoding a protein involved in invasivity and adherence), and ***bib*(A)** (encoding an adhesin) were investigated by PCR using specific primers (Table 1).

Antimicrobial susceptibility testing and macrolide resistance phenotype

The minimal inhibitory concentrations (MICs) were determined using the E-test method according to the protocols established by [12]. The antibiotics tested were: erythromycin, clindamycin, azythromycin, ampicillin, penicillin G and vancomycin. The MLSB resistance phenotype (macrolide–lincosamide–streptogramin B) of the isolates was determined by the double disc diffusion test as described previously[12]. The strain *Streptococcus pneumoniae* ATCC 49619 was used as the quality control.

PCR determination of the macrolide resistance phenotype

The presence of the ***erm*(B), *erm*(C), *erm*(A)** (Subclass ***erm*(TR)**) and ***mef*(A/E)** genes was determined by PCR amplification using the primers described in Table 1. The PCR products were visualized by electrophoresis on 1% agarose gels and sent for sequencing to Beckman Coulter Genomics (United Kingdom).

Statistical analysis

Data related to serotypes, virulence and antimicrobial susceptibility of the 3 study groups were analyzed using contingency tables and Chi-square (x2) tests. A P value < 0.05 was considered statistically significant. The analyses were carried out with the StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP. Correlations of all variables have been determining by the Pearson's correlation coefficient.

RESULTS

Two hundred forty-two GBS **isolates** were included in the study. The **isolates** were divided into 3 groups: colonizers 95/242 (39.3%) (collected from asymptomatic pregnant women), pathogenic 68/242 (28.1%) (collected from symptomatic pregnant women), and neonatal 79/242 (32.6 %) (collected from infected neonates).

Serotype III was the most prevalent (33.9%) followed by serotypes II, Ia, V, IV, Ib, and VII (22.7 %, 14.9%, 10.7%, 8.7%, 8.3% and 0.8% of isolates, respectively). However, serotypes VI, VIII, IX and X were not detected (Table 2). Among the colonizing **isolates**, the most frequent serotypes found were serotype II (31.6%), III (26.3%) and Ia (17.9%), while serotypes III, II and IV (38.2%, 22.1% and 13.2%, respectively) were the most

143 prevalent among the pathogenic **isolates**. In the case of the neonatal **isolates**, serotypes
144 III, Ia and II (39.2%, 22.8% and 12.7%, respectively) were the most frequently found.
145 Serotype Ia was significantly more frequent among colonizing and neonatal **isolates** than
146 among pathogenic **isolates** (p=0.001). On the other hand, serotype II was significantly
147 more frequent among colonizing **isolates** (p=0.012).

148 Regarding the presence of virulence factors, *cyl***(E)** (90.5%), followed by *scp***(B)** (75.6%),
149 *rib* (62.1%) and *bca* (43.4%) (Table 2) were the most frequently found among the
150 **isolates**. The virulence factors *pep* and *bib***(A)** were only found among the neonatal
151 **isolates** (26.6% and 17.7%, respectively). On the other hand, the *bca* and *scp***(B)** genes
152 were significantly more frequent among neonatal **isolates** (68.3% and 87.3%, p=0.0001
153 and 0.002, respectively) and the *fbs***(B)** gene was more frequently found among colonizing
154 (39.1%) and pathogenic **isolates** (39.7%) than among neonatal **isolates** (20.2%) (p=0.01).
155 Significant relationships were observed between the presence of several virulence
156 determinants and serotypes. The *bac* gene was significantly more frequent among
157 serotype Ib (p=0.0002), *rib* gene among serotype III (p= 0.0001), *spb***(1)** gene among
158 serotype II (p= 0.0001), and the *bib***(A)** gene was significantly more frequent among
159 serotype V (p=0.049).
160 All the **isolates** of this study were susceptible to penicillin, ampicillin and vancomycin.
161 However, 21.5% were resistant to azithromycin (MIC>2 μg/mL), 20.7% to erythromycin
162 (MIC>1 μg/mL), and 17.6% to clindamycin (MIC>1 μg/mL). The erythromycin,
163 azithromycin and clindamycin resistant isolates were significantly more frequent among
164 the pathogenic than among the colonizing and neonatal **isolates** (p =0.002, 0.002 and
165 0.001, respectively) (Table 2).
166 Different MLSB phenotypes were observed among the **isolates**; 17.8% of strains showed
167 constitutive resistance to clindamycin (cMLSB), 3.3% showed inducible resistance

(iMLSB) and 0.3% presented the M phenotype. Among the resistant **isolates**, the most prevalent gene found was the ***erm*(B)** gene (40/52, 77 %). 12/52 (23%) **unrelated isolates** showed the combination of genes of ***erm*(A)- *erm*(B)-*mef*(A/E)**; 5/52 (9.7 %) **isolates** presented the ***erm*(A)-*erm*(C)** genes; and 2/52 (3.8 %) **isolates** presented only the ***mef*(A/E)** gene (**Table 3**).

Correlation between serotypes, presence/absence of virulence factors and resistance to antibiotics was analysed. No relationship was observed with antimicrobial resistance. Among colonizing **isolates**, the presence of *bca* gene was strongly associated with the presence of *bac* gene (Pearson coefficient =0.80).

DISCUSSION

S. agalactiae or group B *Streptococcus* (GBS) remains one of the most important etiological causes of neonatal sepsis. However, the real burden of GBS infection in newborns is likely underestimated because some cases are not adequately diagnosed, especially in developing countries [13].

The present study included 242 GBS **isolates** collected from five hospitals in Barcelona. Among the GBS **isolates** studied, four serotypes (III, II, Ia and V) were the most frequent in this study, being serotype II **significantly more prevalent** among colonizing **isolates** and serotype Ia among colonizing and neonatal **isolates**. Our results are somewhat similar to those from other countries in which serotype III was the most frequently found among the GBS strains studied [14, 15]. Another recent report showed that serotype V was the most frequent in France (44.1%) followed by serotype III [16]. In this sense, studies on the distribution of the GBS serotype have shown that GBS varies both geographically and

over time, being serotypes Ia, III, and V more frequently involved in neonatal invasive disease, adult infections and maternal carriage [17, 18].

GBS presented several virulence genes that allow these microorganisms to cause infection and even to cross intact membranes and arrive to the fetus. Recent studies have been focused on analyzing the surface gene profile, but in the present work, other virulence genes such as toxins and invasins were also studied. The most frequent virulence factors found in our study were *cyl***(E)**, *scp***(B)**, *rib* and *bca* most of them related to invasion of epithelial cells [19]. These results are in correlation with previous studies carried out in other geographical areas [7, 20, 21]. In the present work, the *bca* (related to invasion and antophagocytosis) and *scp***(B)** (related to invasion) genes were most prevalent among neonatal **isolates**, probably favoring the colonization and invasion of neonatal mucosa and blood/brain barrier [21]. **In contrast to our findings, Dutra et al.[7] found the *bca* gene more prevalent among colonizing strains and the *scp*(B) gene in the 100% of the strains. The virulence factor *rib* related to invasiveness was more prevalent among serotype III that is associated with invasive infection and, in our study, in agreement with their higher presence in pathogenic isolates. One isolate belonging to serotype III and presenting the *spb*(1) and *rib* virulence genes formed part of the ST17 associated with neonatal invasive infections [23].**

In the present study, we observed a significant percentage of **isolates** resistant to second choice antibiotics, such as azithromycin, erythromycin and/or clindamycin. Remarkably, we found that the percentage of **isolates** resistant to these antibiotics was significantly higher among pathogenic than colonizing and neonatal **isolates** suggesting that, in contrast with other bacteria such *E. coli*, pathogenic **GBS** strains are more **prone** to acquire antimicrobial resistance [24].

Moreover, the rates of resistance to macrolides and clindamycin have also been on the rise worldwide. Domelier *et al.* [25] and Campelo *et al.* [9] reported 19% of resistance to erythromycin among GBS strains causing materno-fetal and neonatal disease in France and Las Palmas (Spain), respectively. On the other hand, a study carried out in Switzerland reported 30% of erythromycin resistance and 28% of clindamycin resistance [26], with a significant increase in the number of multidrug-resistant strains. **However, other studies have found lower rates of isolates resistant to macrolides among pathogenic strains, being lower or equal of 4% [7, 27]. These differences could be due to the selective pressure caused by the antimicrobial treatments that these pregnant women could be received during gestation.**

Interestingly, the resistance to macrolides and lincosamides in GBS in our study was associated with the presence of the ***erm*(B)** gene, being consistent with epidemiological studies about invasive GBS isolates collected from neonates in China and South Korea. On the other hand, the coexistence of several resistant genes such as ***erm(A), erm(B),*** and ***mef*(A/E)** (12 **isolates**) is remarkable in our work. These results differ from those published by other authors, who described combinations of these genes, the most common being the ***erm*(A)/*erm*(B)** and ***erm*(A)/*mef*(A/E)** genes [15, 16, 28].

In contrast with other studies that found a significant relationship between erythromycin resistance and serotype III [29, 30, 31], this association was not present among our isolates.

In conclusion, although penicillin G and ampicillin continue to be a good treatment for GBS infections, a significant increase in the percentage of **isolates** resistant to macrolides and lincosamines has been observed. The fact that this increase was observed mainly among GBS causing symptomatic disease implies a serious problem for treating this type of infections. Surveillance of the epidemiology, antimicrobial resistance and virulence of

241 GBS could provide great knowledge about these microorganisms and help to improve the
242 treatment of infections caused by this opportunistic pathogen.
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