

Multilocus Sequence Types of Invasive and Colonizing Neonatal Group B Streptococci in Poland

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Key Words

Group B streptococcus · Multilocus sequence typing · Newborns · Serotypes · Protein gene profiling · Anti-microbial resistance

Abstract

Objectives: The present study aimed to investigate the molecular characterization of *Streptococcus agalactiae* (group B streptococcus; GBS) strains isolated from newborns with invasive neonatal infections and healthy newborns in Poland. **Materials and Methods:** Forty-two GBS isolates were characterized by combining different typing methods, i.e. multilocus sequence typing (MLST), molecular serotyping and protein gene profiling. **Results:** Using MLST, a total of 16 sequence types (STs) were identified, and among these, 11 were clustered into the following 5 clonal complexes (CCs): CC23 (20; 49%), CC19 (7; 17%), CC17 (4; 10%), CC10 (4; 10%) and CC1 (1; 2%). A statistically significant relationship between ST-17 and invasive isolates ($p = 0.0398$) and ST-23 and colonizing strains ($p = 0.0034$) was detected. Moreover, 2 novel STs were detected (ST-637 and ST-638). Molecular serotyping showed that in the invasive isolates serotype III was predominant (11; 50%), followed by serotypes II (6; 27%), V (3; 14%) and Ia (2; 9%). In healthy newborns, serotype III was also dominant (12; 60%), followed by serotypes Ia (4; 20%), II (2; 10%), V (1; 5%) and Ib (1; 5%). Protein gene profiling indi-

cated that the *rib* gene was predominant in the invasive strains (11; 59%), followed by *bca* (5; 22%), *alp2* (2; 9%), *alp3* (1; 5%) and *epsilon* (1; 5%), while in colonizing strains the *alp2* gene was most common (10; 50%), followed by *epsilon* (5; 25%), *rib* (2; 10%), *bca* (2; 10%) and *alp3* (1; 5%). A statistically significant relationship was noted between the *rib* gene and invasive GBS ($p = 0.0329$), whereas *alp2* was related to the colonizing strains ($p = 0.0495$). **Conclusions:** The investigated GBS isolates originating from infections in newborns and healthy neonates represented serotype III in more than half of the cases and differed from one another in terms of resistance to macrolides, ST type affiliation and the presence of genes encoding surface proteins from the Alp family. Further comparative genetic research on a larger number of strains is necessary for epidemiological investigation and vaccine development.

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Introduction

Streptococcus agalactiae, also referred to as Lancefield group B streptococcus (GBS), is a significant pathogen in neonates and adults with predisposing conditions in the USA and Europe [1, 2]. GBS in newborns can lead to early-onset diseases or late-onset diseases. Early-onset diseases develop during the first 7 days of life, while late-

onset diseases occur after the first week to 90 days of age. The most frequent clinical form of neonatal GBS-caused infection is sepsis or pneumonia; a less frequent one is cerebrospinal meningitis [3].

S. agalactiae colonizes the genitourinary and lower gastrointestinal tract in 10–40% of all pregnant women. Maternal colonization with GBS is the predominant risk factor for the development of invasive neonatal GBS disease [1–3]. GBS strains are subdivided according to type-specific capsular polysaccharides into 10 unique serotypes (Ia, Ib and II–IX) [2, 4]. This capsule has been recognized as one of the major virulence factors with an anti-phagocytic function [1]. The GBS pathogenicity varies between and within serotypes, with considerable variation in genetic content between strains. Particularly, the serotypes most often causing invasive infections in newborns are III, Ia and V [2, 5].

The best-characterized GBS protein antigens, which are significant virulence factors, belong to the alpha-like protein (Alp) family. They are called alpha-C protein, Rib, Alp2, Alp3, Alp4 and epsilon (Alp1) and are encoded by the *bca*, *rib*, *alp2*, *alp3*, *alp4* and *epsilon/alp1* genes, respectively [6]. Examination of the protein gene profile increases the potential for GBS subtyping [7, 8].

Multilocus sequence typing (MLST) has been described to be able to identify the emergence and spread of GBS clones and to study their genetic population structure worldwide [9–12]. Recently, the hypervirulent ST-17 clone, defined with the application of MLST and associated with late-onset diseases and a very high mortality rate in newborns with meningitis, has been of great interest [10–13].

There is limited knowledge regarding the epidemiology and molecular characterization of *S. agalactiae* strains isolated from newborns in Poland and Central-Eastern Europe, mainly due to a lack of supervision of these infections and failure to collect strains derived from neonates. Therefore, the aim of the present study was to determine and comparatively analyse the genotypes of GBS strains isolated from early-onset diseases in newborns and colonized neonates in Poland using different typing methods, such as molecular serotyping, protein gene profiling, antibiotic resistance phenotyping and gene profiling as well as MLST.

Materials and Methods

Study Population and Collection of Specimens

S. agalactiae strains were obtained from the collection of the Department of Bacteriology, Microbial Ecology and Parasitology of Jagiellonian University Medical College (Krakow, Poland) which was created from 2006 to 2010 within the framework of two

projects financed by the Polish Ministry of Research and Higher Education [No. 3PO5E08425 (n = 27) and NN401042337 (n = 15)]. The samples were taken from 22 neonates with early-onset GBS disease as part of standard care, whereas samples from 20 healthy newborns were collected after receiving appropriate consent from their guardians. This study was approved by the Jagiellonian University Bioethical Committee.

The GBS strains originated from 3 neonatal wards from Southern (n = 27), Central (n = 12) and Northern (n = 3) Poland. A single isolate per patient was allowed. Altogether, 42 strains were obtained from newborns aged less than 7 days; 22 of these were from neonates with early-onset GBS disease, and 20 strains were isolated from colonized neonates without any signs or symptoms of infection. In 22 newborns with invasive neonatal infections, blood cultures were performed using Peds Plus Bactec 9120 media (Becton Dickinson, USA). Healthy newborns were tested for GBS colonization by taking swabs from the oral cavity during suction and from the external ear at 24–48 h of age [14]. The samples were placed in a non-nutrient Amies transport medium (Eurotubo, Spain) and within 2–4 h they were inoculated into a selective broth medium, i.e. 3 ml Todd-Hewitt broth (Difco, USA) supplemented with 8 µg/ml gentamicin and 15 µg/ml nalidixic acid (Oxoid, UK), and then incubated overnight at 37°C. Positive blood cultures and pre-incubated selective broth cultures were subcultured onto Columbia 5% sheep blood agar (Difco). The identification of GBS was performed first using a latex agglutination assay and an API STREP kit (bioMérieux, France). Molecular identification based on PCR with Sag59 and Sag190 species-specific primers (Genomed, Poland) for *S. agalactiae* was applied [18]. The tested GBS strains had been previously investigated for the presence of the hypervirulent ST-17 clone as previously described [13].

The characteristics of the serotypes were tested using serological a Group B Streptococcus GBS Serotyping Kit (ESSUM, Sweden). To verify the method, detection of the genes encoding capsular polysaccharides Ia, Ib and II–VIII was investigated using a multiplex PCR method with specific primers (Genomed) according to Poyart et al. [4].

Detection of the surface protein genes *alp2*, *alp3*, *alp4*, *bca*, *epsilon* and *rib* was investigated using a multiplex PCR with specific primers (Genomed) according to the procedure proposed by Creti et al. [7] and Gherardi et al. [2].

For antibiotic resistance, the minimum inhibitory concentrations (MIC) of penicillin, ampicillin, erythromycin, clindamycin and vancomycin were evaluated using Etest (Oxoid) as recommended by EUCAST [15]. The macrolide resistance phenotypes cMLS_B (constitutive), iMLS_B (inducible) and M were determined using a double-disc test with erythromycin (15 µg) and clindamycin (2 µg) (Oxoid). The *erm*(A), *erm*(B), *erm*(C) and *mef*(A/E) resistance determinants were detected by multiplex PCR with 4 adequate pairs of primers (Genomed) according to Sutcliffe et al. [16].

MLST analysis was performed as described by Jones et al. [10] using oligonucleotide primer pairs (Genomed) specific for the 7 housekeeping loci selected for GBS MLST. An online database (<http://pubmlst.org/sagalactiae>) was used to assign alleles to the 7 loci and each isolate was defined by their sequence type (ST). The eBURST program was used to group isolates into clonal complexes (CCs) whose members shared at least 6 of the 7 MLST loci [17]; otherwise, an ST was considered a singleton. Novel alleles of the housekeeping genes were amplified and sequenced twice and these

Table 1. Characteristics of invasive and colonizing GBS strains isolated from neonates according to capsular serotype, Alp family gene and resistance determinant

Investigated features	GBS strain type		Total
	invasive strains	colonizing strains	
Capsular serotype			
Ia	2 (9)	4 (20)	6 (14)
Ib	0 (0)	1 (5)	1 (2)
II	6 (27)	2 (10)	8 (19)
III	11 (50)	12 (60)	23 (55)
V	3 (14)	1 (5)	4 (10)
Total	22 (100)	20 (100)	42 (100)
Gene from the Alp family			
<i>alp2</i>	2 (9)	10 (50) (p = 0.0495)*	12 (28)
<i>alp3</i>	1 (5)	1 (5)	2 (5)
<i>bca</i>	5 (23)	2 (10)	7 (17)
<i>epsilon</i>	1 (5)	5 (25)	6 (14)
<i>rib</i>	13 (58)	2 (10) (p = 0.0329)*	15 (36)
Total	22 (100)	20 (100)	42 (100)
Erythromycin resistance gene			
<i>ermB</i>	6 (27)	0 (0) (p = 0.0038)*	6 (14)
Total	22 (100)	20 (100)	42 (100)

Values represent n (%). * Statistically significant (p < 0.05).

profiles were named 'new' and added to the MLST database with a new ST number.

The potential relationship between invasive or colonizing GBS isolates and STs, CCs or serotypes was analysed.

Statistical Analysis

For statistical analysis of the frequencies and probability of coincidence, a χ^2 test was used. When data were insufficient for the test demands, a G² (likelihood ratio test) was used. Comparison of the antibiotic resistance between invasive and colonizing strains was made with a Wilcoxon test. p < 0.05 was considered statistically significant. Analyses were conducted using the SAS 9.1 package and SAS Enterprise Guide 3.0 (SAS Institute Inc., Cary, N.C., USA).

Results

Serotypes of Neonatal GBS Strains

In the group of 22 invasive GBS isolates, serotype III was predominant (11; 50%), followed by serotypes II (6; 27%), V (3; 14%) and Ia (2; 9%). In the 20 healthy newborns colonized by GBS, serotype III was also predominant (12; 60%), followed by serotypes Ia (4; 20%), II (2; 10%), V (1; 5%) and Ib (1; 5%). In the study population,

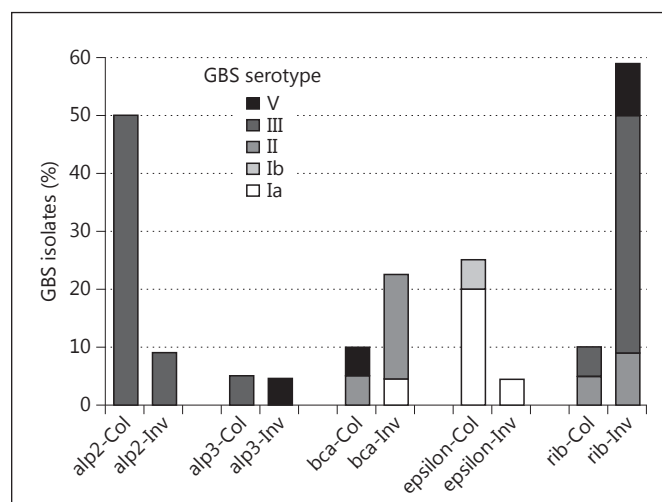


Fig. 1. Diversity of surface protein genes from the Alp family among the particular serotypes in GBS strains isolated from newborns with invasive neonatal infections and healthy newborns. Col = Colonizing GBS; Inv = invasive GBS.

serotypes IV and VI–VIII were not identified. No significant differences in serotype distribution were noted between the study groups of newborns (table 1).

Surface Protein Genes of Neonatal GBS Strains

One type of surface protein gene from the Alp family was found in each strain. In the invasive GBS isolates, *rib* was predominant (13; 58%), followed by *bca* (5; 23%), *alp2* (2; 9%), *alp3* (1; 5%) and *epsilon* (1; 5%). The most common gene in the colonizing GBS strains was *alp2* (10; 50%), followed by *epsilon* (5; 25%), *rib* (2; 10%), *bca* (2; 10%) and *alp3* (1; 5%). In this population of GBS isolates, the *alp4* gene was not found. Moreover, the relationships between the capsular polysaccharide serotype and surface protein genes in invasive and colonizing isolates were noted (fig. 1). A statistically significant relationship was observed between the *rib* gene and invasive GBS strains with the III serotype ($\chi^2 = 4.549$; p = 0.0329) and the *alp2* gene and colonizing GBS isolates with the III serotype ($\chi^2 = 3.630$; p = 0.0495) (table 1).

Resistance of the GBS Strains

The values of MIC₅₀ and MIC₉₀ for penicillin, ampicillin, erythromycin, clindamycin and vancomycin are given in table 2. All GBS isolates were susceptible to penicillin, ampicillin and vancomycin. Resistant strains were found only in neonates with infections; 6 (27%) of these invasive isolates were resistant to erythromycin (G² =

Table 2. Distribution of MIC in invasive and colonizing GBS strains from neonates

Antibiotics	MIC	MIC for colonizing strains (n = 20), µg/ml	MIC for invasive strains (n = 22), µg/ml
Penicillin	MIC ₅₀	0.06	0.06
	MIC ₉₀	0.12	0.12
Ampicillin	MIC ₅₀	0.06	0.06
	MIC ₉₀	0.078	0.06
Erythromycin	MIC ₅₀	0.06	0.06
	MIC ₉₀	0.06	256 (p = 0.0380)*
Clindamycin	MIC ₅₀	0.06	0.06
	MIC ₉₀	0.06	256 (p = 0.0090)*
Vancomycin	MIC ₅₀	0.5	0.5
	MIC ₉₀	1	1

* Statistically significant (p < 0.05).

8.356; p = 0.0038) and 5 (23%) were resistant to clindamycin ($G^2 = 6.823$; p = 0.0090). Macrolide resistance was not attributable to a single serotype, ST or CC. Among the 6 resistant strains, the cMLS_B phenotype was detected in 5 (83%) cases and iMLS_B in 1 (17%). The *ermB* gene was present in all 6 macrolide-resistant strains. The *ermA*, *ermC* and *mef(A/E)* genes were not detected in the collected strains.

MLST Analysis

A total of 16 STs were identified for 42 of the examined strains. Among these, 11 were clustered into 5 CCs, the most prevalent of which was CC23 (20; 49%), followed by CC19 (7; 17%), CC17 (4; 10%), CC10 (4; 10%) and CC1 (1; 2%). The singletons identified were ST-22, ST-255 and ST-410; moreover, 2 novel STs were detected, i.e. ST-637 and ST-638 (table 3).

A statistically significant variability in the number of STs in invasive isolates (n = 13) in relation to the colonizing strains (n = 6) was noted ($\chi^2 = 10.3$; p = 0.0013). A diverse population was found among the GBS isolates, with some exceptions, i.e. that ST-17 occurred only in invasive isolates with serotype III while ST-23 was more frequent in colonizing strains with serotype III (table 3; fig. 2).

Variability in the serotype distribution was observed within some of the STs. For instance, of the 16 isolates identified as ST-23, 11 were serotype III and 5 were sero-

type Ia. Similarly, of the 5 isolates identified as ST-19, 3 were serotype II and 2 were serotype III. Conversely, all ST-17 isolates represented serotype III, and all ST-12 isolates belonged to serotype II.

Discussion

Our study demonstrated that the population structure of GBS isolates in Polish neonates is similar to the GBS population structures in other European countries and around the world [18, 19]. *S. agalactiae* is a genetically diverse organism. Therefore, a combination of several molecular typing methods should be considered to provide a better understanding of the pathogenesis and epidemiology of GBS isolates [20]. Numerous epidemiological investigations have shown that the serotype distribution of GBS varies both geographically and over time [2, 4, 5]. In our study, serotype III was predominant in both study groups of neonates and represented 50% of the invasive GBS isolates and 60% of isolates from newborn colonization. Other serotypes in invasive strains were in sequence II, V and Ia and for colonizing strains Ia, II, V and Ib. When comparing the present results with previous investigations on 169 GBS-positive pregnant women in whom serotype III was predominant in 35% of cases, followed by Ia, V, II, Ib and IV, we concluded that the current reported results are quite similar but with varying degrees of participation of individual serotypes [8]. The data obtained in relation to the serotype distribution correspond with the results of other European countries where serotypes Ia, II, III and V have been found in 80–90% of all clinical isolates, while serotypes VI and VII–IX have seldom been observed [2, 5]. Fluegge et al. [21] noted that serotype III was the most common in invasive and non-invasive GBS strains isolated from newborns, and it was significantly predominant in invasive isolates.

Surface protein antigens play an important role in the pathogenesis of GBS infection, and several of these antigens have been suggested as components of GBS conjugate vaccines [1, 6]. Protein gene profiling increases the potential for GBS subtyping [7]. The association of the *rib* gene and serotype III has been confirmed by several reports [2, 21]. In our study, the percentages of *rib*-positive isolates within serotype III strains varied significantly; in invasive isolates, the *rib* gene was detected in 59% of cases, while in colonizing strains *rib* was identified in 10% of cases. These results agree with previous studies which demonstrated that the *rib* gene was associated with invasive strains [22]. These findings may be evidence of an

Table 3. Characteristic of the CCs and STs of invasive and colonizing GBS strains isolated from neonates according to virulence factors (serotypes and Alp genes) and resistance determinants (*ermB* gene)

CC	ST	Strain type, n		Capsular serotype	Gene from the Alp family	Erythromycin resistance determinant
		colonizing	invasive			
CC1 (1)	ST-1 (1)	0	1	V (1)	<i>alp3</i> (1)	
CC10 (4)	ST-10 (1)	0	1	II (1)	<i>bca</i> (1)	
	ST-12 (2)	1	1	II (2)	<i>bca</i> (2)	
	ST-358 (1)	0	1	III (1)	<i>rib</i> (1)	<i>ermB</i> (1)
CC17 (4)	ST-17 (4)	0	4 (p = 0.0398)*	III (4)	<i>rib</i> (4)	
CC19 (7)	ST-19 (5)	2	3	II (3) III (2)	<i>rib</i> (5)	<i>ermB</i> (2)
	ST-106 (1)	0	1	II (1)	<i>bca</i> (1)	
	ST-286 (1)	0	1	III (1)	<i>rib</i> (1)	
CC23 (20)	ST-23 (16)	13	3 (p = 0.0034)*	Ia (5) III (11)	<i>alp2</i> (10) <i>alp3</i> (1) <i>epsilon</i> (5)	
	ST-220 (3)	0	3	Ia (1) III (1) V (1)	<i>bca</i> (1) <i>rib</i> (2)	
	ST-249 (1)	1	0	III (1)	<i>alp2</i> (1)	
Singleton (5)	ST-22 (1)	0	1	II (1)	<i>bca</i> (1)	<i>ermB</i> (1)
	ST-255 (1)	1	0	Ib (1)	<i>epsilon</i> (1)	
	ST-410 (1)	0	1	III (1)	<i>rib</i> (1)	<i>ermB</i> (1)
	ST-637 (1)**	1	0	V (1)	<i>bca</i> (1)	
	ST-638 (1)**	0	1	V (1)	<i>rib</i> (1)	<i>ermB</i> (1)
Not tested (1)	NT (1)	1	0	III (1)	<i>alp2</i> (1)	

Numbers in parentheses represent the number of isolates. * Statistically significant (p < 0.05). ** New sequence type.

important role of the Rib protein in invasive disease [21, 23]. Moreover, a statistically significant relationship between *alp2*-positive isolates within serotype III strains and colonizing status was detected. The noted relationship is interesting and has not been described by other investigators. In contrast, the most common Alp gene in GBS strains isolated from pregnant women from our previous report was *epsilon*, and it accounted for 26% of all cases [8].

For many years, GBS remained susceptible to penicillin, the recommended drug for the treatment and prophylaxis of infections caused by GBS. In the USA, for patients allergic to penicillin, the alternative drugs according to the 2002 recommendation of the Centers for Disease Control and Prevention (CDC) were macrolides and lincosamides [24]. However, in 2006 in the USA, 24.5% of invasive and 41.9% of colonizing GBS isolates

were erythromycin resistant [26]. Due to this high level of erythromycin resistance, the CDC did not recommend erythromycin anymore in 2010 [25]. In Europe, the erythromycin resistance in GBS isolates from newborns and adults ranged from 10% in Portugal [27] to 16% in Poland [28] and 16.5% in Italy [2]. In the examined population of newborns, resistant strains were found only in neonates with invasive infections; 27% of these were resistant to erythromycin and 23% were resistant to clindamycin. Our results indicate a very high percentage of resistant strains in the study group of neonates.

Two principal resistance mechanisms are described for GBS: methylation of 23S ribosomal RNA, determined by *erm* genes, and active drug efflux by pumps encoded by *mef* genes. Expression of *erm* genes is described as the MLS_B phenotype (cMLS_B constitutive; iMLS_B inducible), while expression of *mef* genes is called the M phenotype

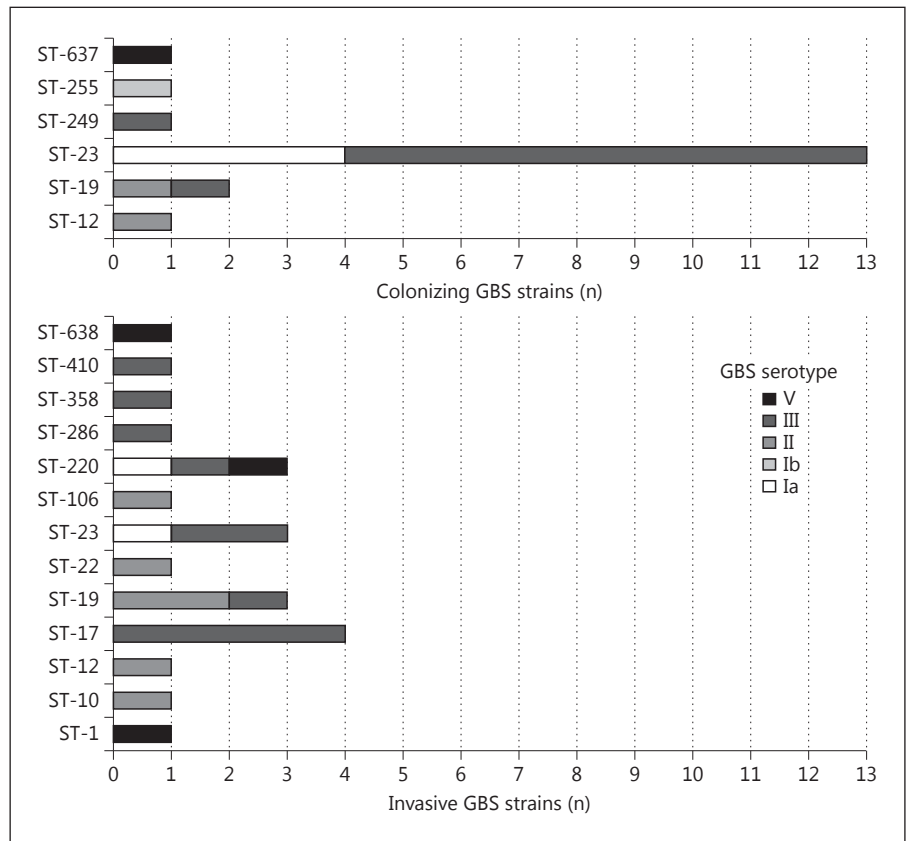


Fig. 2. Diversity of STs among particular serotypes in GBS strains isolated from newborns with invasive neonatal infections and healthy newborns.

[2]. Among our invasive strains, the $cMLS_B$ phenotype was predominant in 83% of cases, followed by $iMLS_B$ in 17%, and the presence of the *ermB* gene was detected in all of the cases. Similar results have been noted by other investigators. For example, Sadowy et al. [9] reported that the most prevalent determinant of resistance was *ermB*, usually located on the Tn3872-like transposon.

MLST has become the conventional method for determining the population structure of GBS, and it has been applied to the molecular epidemiology of *S. agalactiae* infections by several investigators [9, 10, 19]. To date, 5 main CCs, i.e. CC1, CC10, CC23, CC19 and CC17, have been identified, emphasizing the diversity of *S. agalactiae* in humans [9, 19]. In our study, approximately 86% of the invasive isolates and 85% of the colonizing strains could be grouped into 1 of the 5 CCs (CC23, CC19, CC17, CC10 or CC1). These 5 CCs have also been highly widespread in other GBS collections, i.e. from Italy [2], Sweden [29], the USA [19] and Taiwan [30]. Sadowy et al. [9] reported that GBS isolates represented 30 different STs grouped into 4 CCs, i.e. CC1, CC17, CC19, and CC23, that constituted 98.2% of all the studied isolates.

In our study, CC17 occurred only in invasive isolates with serotype III and *rib*, while CC23 was more frequently present in colonizing strains with serotype III and *alp2*. Similarly, a study by Bohnsack et al. [19] demonstrated that CC17 was strongly correlated with serotype III and invasive infection, whereas CC23 strains were predominant in 98% of cases among serotype Ia strains that colonize human neonates. In contrast, in a German-based study, MLST of non-invasive serotype III isolates from neonates in whom sepsis was suspected but whose blood cultures were sterile revealed that ST-389, which belongs to CC19, was predominant [21].

The hypervirulent clone ST-17 represents successful invasive clones which belong to the complex CC17 and are responsible for causing invasive infections in newborns, and it is now considered the most important type of ST worldwide [12, 13, 18, 29]. This clone is isolated from neonates with both early-onset diseases and late-onset diseases and the majority of cases present in the form of meningitis, with a high mortality rate (70%) [19, 29]. Straková et al. [12] identified the ST-17 clone in 85% of cerebrospinal fluid isolates (6 out of 7) but only in 32%

of blood isolates. In our study, the hypervirulent clone ST-17 was detected in 4 cases (18%) of early sepsis developing during the first day of life.

Similarly to the findings of Luan et al. [29], within some of the STs, we noticed variability in the serotype distribution. For example, out of the 16 isolates identified as ST-23, 11 were serotype III and 5 were serotype Ia; moreover, 3 out of 3 ST-220 isolates expressed 3 different serotypes, i.e. Ia, III and V. These results confirmed that the horizontal transfer of capsular genes exists in the GBS population. Indeed, the level of capsular switching appears to be high in *S. agalactiae* strains and the exchange of capsule polysaccharide genes between isolates with different genetic backgrounds occurs without restrictions to certain genetic lineages [29].

A limitation of our study is the small number of collected strains from neonates. However, due to a complete lack of similar analyses from our country, we strongly believe that our results are unique from the Polish perspective and support important information about neonatal GBS isolates. Admittedly, Sadowy et al. [9] conducted studies on the population structure of

GBS in Poland, but the analysis involved strains isolated from different clinical materials, mainly from adults.

Conclusion

The investigated GBS isolates originating from infections in newborns and healthy neonates represented serotype III in more than a half of the cases and differed from one another in terms of resistance to macrolides, ST type affiliation and the presence of genes encoding surface proteins from the Alp family. Further comparative genetic research on a larger number of strains is necessary for epidemiological investigation and vaccine development.

Acknowledgements

This study was supported by a grant from the Polish Ministry of Research and Higher Education (No. N N401 042 337). This study was approved by Jagiellonian University Bioethical Committee decision No. KBET/143/B/2007.

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