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Genetic characterization and diversity of *Streptococcus agalactiae* isolates with macrolide resistance

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Macrolide resistance in 169 *Streptococcus agalactiae* [group B streptococcus (GBS)] isolates originating from pregnant carriers was investigated. Using multiplex PCR the presence of genes encoding erythromycin resistance and capsular polysaccharides, as well as surface proteins, was determined. Random amplification of polymorphic DNA (RAPD) and PFGE were used to characterize specific clones among the isolates. In the examined population of women, erythromycin-resistant strains were found in 4.5 % of patients, whereas clindamycin-resistant strains were found in 3 % of patients, which was 16 % of strains resistant to erythromycin and 10 % of strains resistant to clindamycin among GBS isolates, respectively. Among the isolates, the largest percentage was represented by the constitutive macrolide–lincosamide–streptogramin B ($cMLS_B$) phenotype (63 %), then the inductive macrolide–lincosamide–streptogramin B ($iMLS_B$) phenotype (26 %) and the macrolide resistance (M) phenotype (11 %). The *ermB* gene was indicated in all isolates with the $cMLS_B$ phenotype and V serotype, whereas *mefA/mefE* genes were found in isolates with the M phenotype and Ia serotype. Among resistance isolates, serotype V was predominant (67 %), followed by serotypes II (15 %), Ia (11 %) and III (7 %). The most common surface protein encoding genes were *alp3* (70 %), then *rib* (11 %), *epsilon* (7.5 %), *bca* (7.5 %) and *alp2* (4 %). A statistically significant relationship between macrolide resistance, serotype V and the *alp3* gene was demonstrated. PFGE, in comparison to the RAPD method, gave better genetic discrimination of GBS isolates. A relatively high genetic diversity among investigated strains was shown. In addition, the largest genetic homogeneity was found in serotype V.

INTRODUCTION

Streptococcus agalactiae [group B streptococcus (GBS)], despite the fact that antibiotic prophylaxis is implemented during the perinatal period in the USA and some European countries, is still one of the major aetiological causes of sepsis and newborn meningitis. Moreover, in recent years, GBS has been isolated more often from immunosuppressed patients (Gherardi *et al.*, 2007; Schrag *et al.*, 2002).

Penicillin is the recommended treatment drug, to which no case of GBS resistance has been reported. However, for people allergic to penicillin, the alternative drugs are macrolides (e.g. erythromycin) or lincosamides (e.g. clindamycin) (Heczko *et al.*, 2008; Schrag *et al.*, 2002).

Abbreviations: $cMLS_B$, constitutive macrolide–lincosamide–streptogramin B; CPS, capsular polysaccharides; GBS, group B streptococcus; $iMLS_B$, inductive macrolide–lincosamide–streptogramin B; RAPD, random amplification of polymorphic DNA.

Two main resistance mechanisms have been described for *S. agalactiae*. *erm* (erythromycin ribosome methylase) genes such as *ermA* and *ermB* (subclass *ermTR*), encode methylase 23S rRNA, which is responsible for methylation of erythromycin and clindamycin receptor sites in ribosomes. Expression of these genes is described as the MLS_B phenotype and points to cross-resistance to macrolides, lincosamides and streptogramin B. This resistance can be constitutive macrolide–lincosamide–streptogramin B ($cMLS_B$) resistance, as well as inductive macrolide–lincosamide–streptogramin B ($iMLS_B$) resistance. Genes *mefA* and *mefE* [macrolide resistance (M) phenotype] are responsible for the second mechanism, in which highly conservative sequences encode a pump that expels antibiotics from inside of the bacterial cell. Expression of these genes is described as the M phenotype (Gherardi *et al.*, 2007).

Epidemiological analyses of GBS isolates are mainly based on capsule serotyping. Currently, ten different GBS

serotypes have been described: Ia, Ib and II–IX. Serotype distribution varies with geographical region, ethnic origin and the virulence of clinical isolates (Persson *et al.*, 2004; Poyart *et al.*, 2007; Slotved *et al.*, 2007).

GBS can also be classified on the basis of surface protein antigens. The major surface-localized protein antigens of GBS belong to a family of surface proteins and are the alpha-C protein, rib, alpha-like protein 2, alpha-like protein 3, alpha-like protein 4 and epsilon protein (alpha-protein-like proteins) that are encoded by *bca*, *rib*, *alp2*, *alp3*, *alp4* and *epsilon* genes, respectively. The possibility of looking at the protein gene profile increases the potential of GBS subtyping (Creti *et al.*, 2004; Persson *et al.*, 2008).

Genotyping methods, including the random amplification of polymorphic DNA (RAPD) method, as well as PFGE, are used to characterize and distinguish specific clones among GBS isolates (Martinez *et al.*, 2000; Skjaervold *et al.*, 2004).

Because in recent years the resistance of *S. agalactiae* to macrolides, lincosamides and streptogramin B is being reported more often (Acikgoz *et al.*, 2004; Chohan *et al.*, 2006; De Mouy *et al.*, 2001; Hsueh *et al.*, 2001; Schoening *et al.*, 2005), from an epidemiological point of view it is important to conduct detailed characteristics of these isolates. These studies were performed in few specialized scientific centres around the world (Diekema *et al.*, 2003; Domelier *et al.*, 2008; Gherardi *et al.*, 2007; Uh *et al.*, 2005), yet there are no Polish data whatsoever. Therefore, the aims of our studies were: (i) to determine the frequency of the occurrence of resistance phenotypes to macrolides in *S. agalactiae* isolates originating from pregnant women; (ii) to show correlation between the occurrence of $cMLS_B$, $iMLS_B$ or M phenotype and the presence of genes encoding resistance to erythromycin, as well as genes coding capsular polysaccharides (CPS) and surface proteins; (iii) to conduct genetic similarity tests of GBS isolates using PFGE and compare the results to RAPD.

METHODS

Research on the macrolide resistance of *S. agalactiae* isolates was carried out from August 2007 until September 2008. A total of 601 women in the third trimester of pregnancy from Southern Poland underwent examination of GBS colonization according to Centers for Disease Control and Prevention recommendations (Brzychczy-Włoch *et al.*, 2008b; Heczko *et al.*, 2008; Schrag *et al.*, 2002). The study was approved by the Jagiellonian University Bioethical Committee, decision no. KBET/143/B/2007.

Macrolide resistance in 169 GBS isolates originating from pregnant carriers was investigated. The disc diffusion method, with clindamycin (2 μg) and erythromycin (15 μg) (Oxoid), as well as Etests (AB Biodisk), were used. Phenotypes $cMLS_B$, $iMLS_B$ and M were detected. The results were interpreted according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2009).

Analysis of the genes for resistance to erythromycin *ermA*, *ermB*, *ermC* and *mefA/mefE* was carried out using PCR with four pairs of primers (Genomed) according to Sutcliffe *et al.* (1996), as previously described (Brzychczy-Włoch *et al.*, 2008a). The characteristics of CPS were tested serologically using a group B streptococcus GBS serotyping kit (ESSUM). To verify the method, the detection of genes encoding particular CPS was performed using a multiplex PCR method, according to Poyart *et al.* (2007).

A multiplex PCR method was used to detect the surface protein genes *bca*, *rib*, *epsilon*, *alp 2/3* and *alp4* with five pairs of primers (Genomed) according to the procedure of Creti *et al.* (2004). To distinguish between *alp2/3* and confirm presence of Alp3 protein, the reverse primer Alp3 (5′-TTT TGG TTC GTT GCT ATC CTT AAG C-3′) was used according to Gherardi *et al.* (2007), and the universal forward primer was used according to Creti *et al.* (2004). The PCR products were analysed in 1.5 % agarose gel in the presence of ethidium bromide (0.25 μg ml^{-1}) (Bio-Rad).

The analysis of the genetic similarity of GBS isolates resistant to macrolides was performed using RAPD and PFGE methods. RAPD was performed with primer OPB17 (Genomed) according to Martinez *et al.* (2000), as previously described (Brzychczy-Włoch *et al.*, 2008a). PFGE was performed according to Tynkkynen *et al.* (1999), with the following modifications. Bacteria were cultured in tryptic soy broth (Difco) at 37 °C for 24 h and 25 U restriction enzyme *Sma*I (Fermentas) was used. PFGE was performed using the CHEF-DR III device (Bio-Rad), with the following parameters: pulses 5–40 s; 6 V cm^{-1}; 14 h; 14 °C. The comparison of the genetic profiles obtained from RAPD and PFGE was carried out with Molecular Analyst (Applied Maths) software.

The final pictures from all electrophoresis were analysed using QuantityOne software (Bio-Rad), as well as a GelDoc2000 device (Bio-Rad).

For statistical analysis the χ^2 test was used. In the case of small sample sizes Fisher's exact test was used. When the frequency was zero, the G^2 (likelihood ratio) test was used. P values of <0.05 were considered significant.

RESULTS AND DISCUSSION

The increasing resistance to macrolides among group B streptococci observed in recent years in many countries is a therapeutic problem among patients allergic to β-lactams (Acikgoz *et al.*, 2004; Chohan *et al.*, 2006; De Mouy *et al.*, 2001; Hsueh *et al.*, 2001; Schoening *et al.*, 2005). Because of this fact there is a need to monitor the phenomenon and implement rational antibiotic therapy. From the epidemiological point of view, it is essential to perform specific characterization of GBS isolates, to find, among others, an answer to whether the observed phenomenon occurred due to the spread of a specific GBS clone in the population, or whether it is the result of acquired resistance among *S. agalactiae* strains.

In 2008 in Poland, recommendations were developed to screen pregnant women for GBS colonization and, if needed, implement perinatal antibiotic prophylaxis, according to the Centers for Disease Control and Prevention recommendations (Schrag *et al.*, 2002). Antibiotic therapy used on a wide scale may cause an increase in the resistance of GBS isolates, as has happened in other countries. A good example of this is the USA, where the percentage of GBS isolates resistant to erythromycin in the

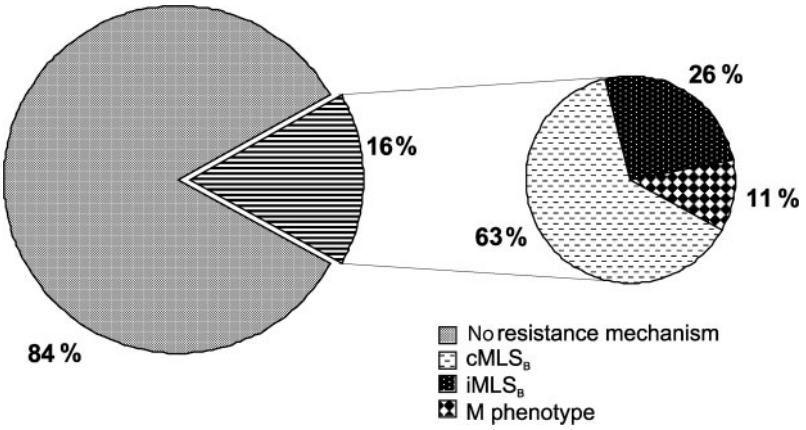

Fig. 1. Distribution of *S. agalactiae* isolates (n=27) with cMLS$_B$, iMLS$_B$ and M resistance phenotypes among pregnant women carriers (n=169).

1980s century was 1.2 %, in the 1990s was 18 % and at the beginning of 21st century it reached 40 %; similarly in Asia, where resistance to macrolides has now reached up to 50 % (De Mouy *et al.*, 2001; Hsueh *et al.*, 2001).

In Europe the percentage of GBS resistant to erythromycin, according to available literature, is: 11 % in Germany (Schoening *et al.*, 2005), 14 % in Spain (Acikgoz *et al.*, 2004), 16 % in Italy (Gherardi *et al.*, 2007), 21 % in France (De Mouy *et al.*, 2001) and 22 % in Turkey (Acikgoz *et al.*, 2004).

In our study, the susceptibility to erythromycin and clindamycin was determined for isolates of *S. agalactiae* originating from pregnant women carriers. The frequency of occurrence of GBS strains resistant to erythromycin was determined to be 4.5 % and to clindamycin to be 3 % of the examined female population (n=601), which turned out to be 16 % (n=27) of strains resistant to erythromycin and 10 % (n=17) of strains resistant to clindamycin among GBS isolates (n=169), respectively. In general the phenotypic mode of resistance to macrolides was shown for 27 isolates, among which the cMLS$_B$ phenotype (n=17, 63 %) was most common, then the iMLS$_B$ phenotype (n=7, 26 %) and the M phenotype (n=3, 11 %) (Fig. 1). Using Etest the values for MIC$_{50}$ and MIC$_{90}$ were determined for erythromycin and clindamycin (Table 1).

The data obtained correspond with the results of a French study, where 70 % of isolates had the cMLS$_B$ phenotype, 22 % the iMLS$_B$ phenotype and about 8 % the M phenotype (De Mouy *et al.*, 2001), and partially with outcomes of a German research group, where cMLS$_B$ dominated accounting for 41 % of isolates, iMLS$_B$ for 38 % and M for 21 % (Schoening *et al.*, 2005). Slightly different data come from Asia, where in almost 90 % of the analysed phenotypes, the constitutive type of resistance was predominant (Hsueh *et al.*, 2001). Quite different results were described in Turkey, where the iMLS$_B$ phenotype dominated at 80 %, while cMLS$_B$ was present at only 20 % (Acikgoz *et al.*, 2004).

Among investigated GBS isolates with a phenotypically confirmed mechanism of resistance to macrolides, the detection of *ermA*, *ermB*, *ermC*, *mefA/mefE* genes encoding resistance to erythromycin was performed. The *ermB* gene dominated and its presence was shown for all strains with the cMLS$_B$ phenotype, and belonging to V serotype (n=17), while *mefA/mefE* genes were shown in isolates with the M phenotype, representing Ia serotype (n=3). The presence of *ermA* and *ermC* genes was not detected in any of the tested strains (Table 1). For our study of the occurrence of genes encoding resistance to erythromycin, similar results were obtained to these found in the literature (De Mouy *et al.*, 2001; Gherardi *et al.*, 2007; Hsueh *et al.*, 2001; Uh *et al.*, 2005).

The detection of genes encoding particular CPS was performed with multiplex PCR. Among strains resistant

Table 1. Association of phenotypes, MICs of erythromycin and clindamycin, and genotypes

Resistance phenotype (n)	Susceptibility result	EM		CM		Erythromycin resistance gene
		MIC$_{50}$ (μg ml^{-1})	MIC$_{90}$ (μg ml^{-1})	MIC$_{50}$ (μg ml^{-1})	MIC$_{90}$ (μg ml^{-1})	
cMLS$_B$ (17)	EM, r/i; CM, r/i	>256	>256	>256	>256	*ermB* (n=17)
iMLS$_B$ (7)	EM, r/i; CM, s (D zone)	1.75	3.25	0.32	0.66	–
M phenotype (3)	EM, r/i; CM, s	3.0	3.5	0.07	0.08	*mefA/mefE* (n=3)

CM, Clindamycin; EM, erythromycin; i, intermediate; r, resistant; s, sensitive.

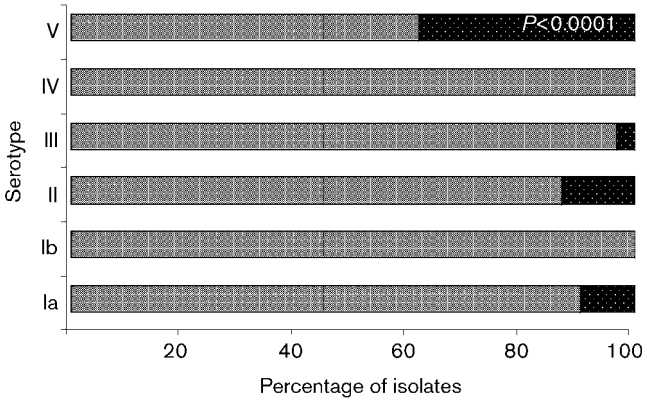

Fig. 2. Correlation of macrolide resistance with serotype of *S. agalactiae* isolates (n=169). Grey bars, no resistance mechanism; black bars, resistance mechanism.

to macrolides, the V serotype (n=18, 67 %) dominated, then II (n=4, 15 %), Ia (n=3, 11 %) and III (n=2, 7 %). In this pool of isolates none of the other serotypes were found. Based on data collected for all the studied women, showing the distribution of serotypes among *S. agalactiae* isolates (n=169), the expected numbers of resistant strains that represented particular serotypes were calculated, then checked if they were far different from obtained values. We showed a statistically significant relationship between resistance to macrolides and the V serotype of *S. agalactiae*; however, there was not a similar statistically significant relationship with the other serotypes (Fig. 2).

At the turn of the last decade there were more frequent reports of the isolation of *S. agalactiae* strains belonging to serotype V, which in most cases were isolated from different types of infections, especially from older or immunosuppressed people (Gherardi *et al.*, 2007). Nowadays, we think that the spread of the V serotype in the population is one of the more serious threats related to GBS epidemiology. Uh *et al.* (2005) showed 60 % domination of V serotype in GBS strains resistant to

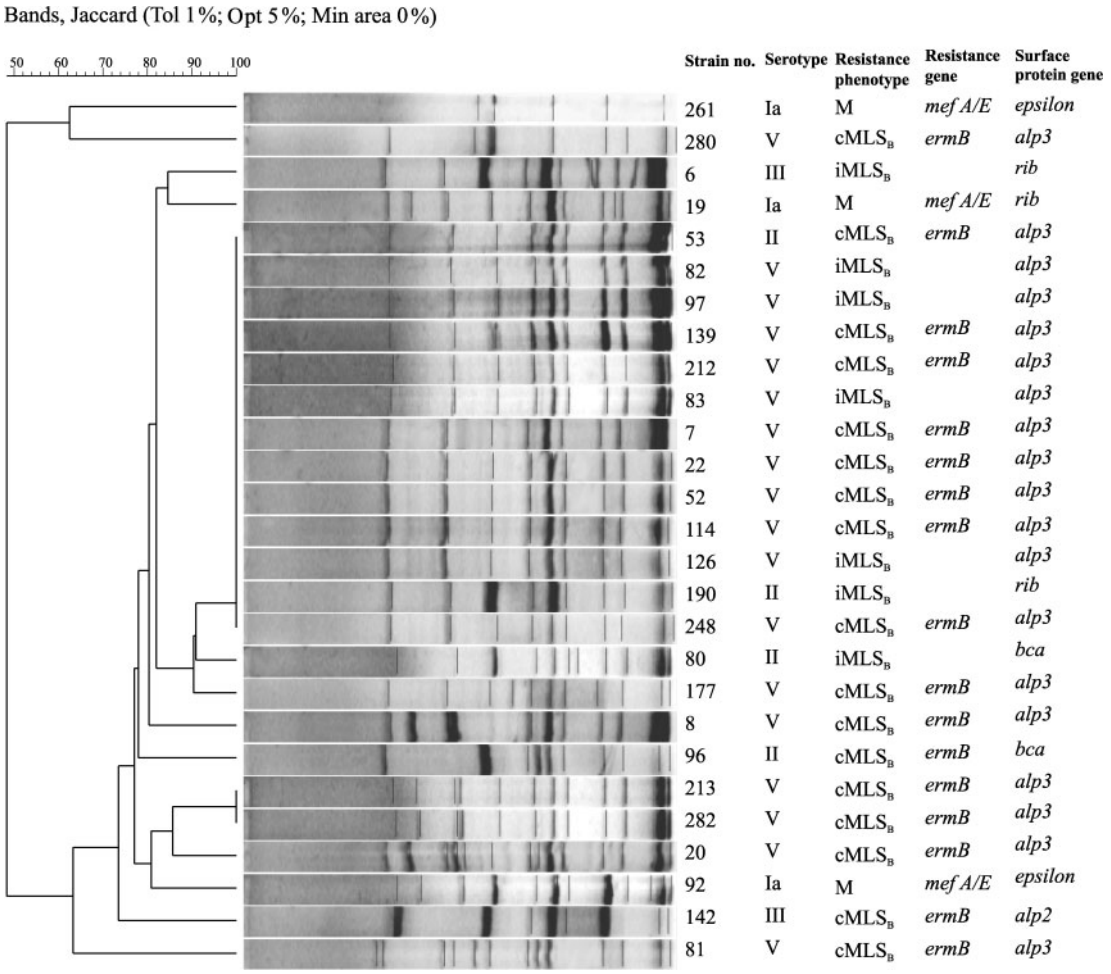

Fig. 3. Genetic similarity analysis using RAPD for 27 *S. agalactiae* isolates with the macrolide-resistance phenotype.

macrolides and similar results were also reported by European centres (Diekema *et al.*, 2003; Gherardi *et al.*, 2007). Our results are consistent with the literature and point to a dangerous phenomenon in recent years, which is an increasing number of GBS isolates being resistant to erythromycin, representing serotype V.

The surface proteins of GBS are likely to play an important role in the pathogenesis of *S. agalactiae* infection (Creti *et al.*, 2004; Persson *et al.*, 2008). Creti *et al.* (2004) noted a relationship between serotypes and surface protein genes, but to a lesser degree than previously reported. They found the association of serotypes Ia, Ib and II with the alpha-C protein, of serotype III with Rib, and serotypes V and VIII with Alp3, but it was not absolute (Creti *et al.*, 2004). Among macrolide-resistant isolates investigated in the present study, the *alp3* gene significantly dominated (n=19, 70 %). In this pool of isolates we found *rib* (n=3, 11 %), *epsilon* (n=2, 7.5 %), *bca* (n=2, 7.5 %) and *alp2* (n=1, 4 %) genes too, but in lesser degree, and no *alp4* were present.

Methods used to perform genetic similarity analysis between GBS isolates are, among others, RAPD as well as PFGE (Gherardi *et al.*, 2007; Martinez *et al.*, 2000). The great advantage of RAPD is a relatively short procedure time and simple methodology. This method was successfully used in analysing genetic isolates of *S. agalactiae* both in the study by Martinez *et al.* (2000) and in our previous study (Strus *et al.*, 2009).

In this study a better genetic discrimination of GBS isolates was shown thanks to PFGE, where 20 pulsotypes were obtained for 27 isolates, in comparison to RAPD where 14 genotypes were obtained for 27 isolates (Figs 3 and 4). Special attention should be given to the cluster obtained with RAPD, comprising 13 GBS isolates (strain numbers: 53, 82, 97, 139, 212, 83, 7, 22, 52, 114, 126, 190, 248), which were genetically identical (Fig. 3). The use of PFGE allowed differentiation of these 13 strains into 6 different pulsotypes (Fig. 4). Comparing outcomes of PFGE with RAPD results allows us to conclude that PFGE is a more

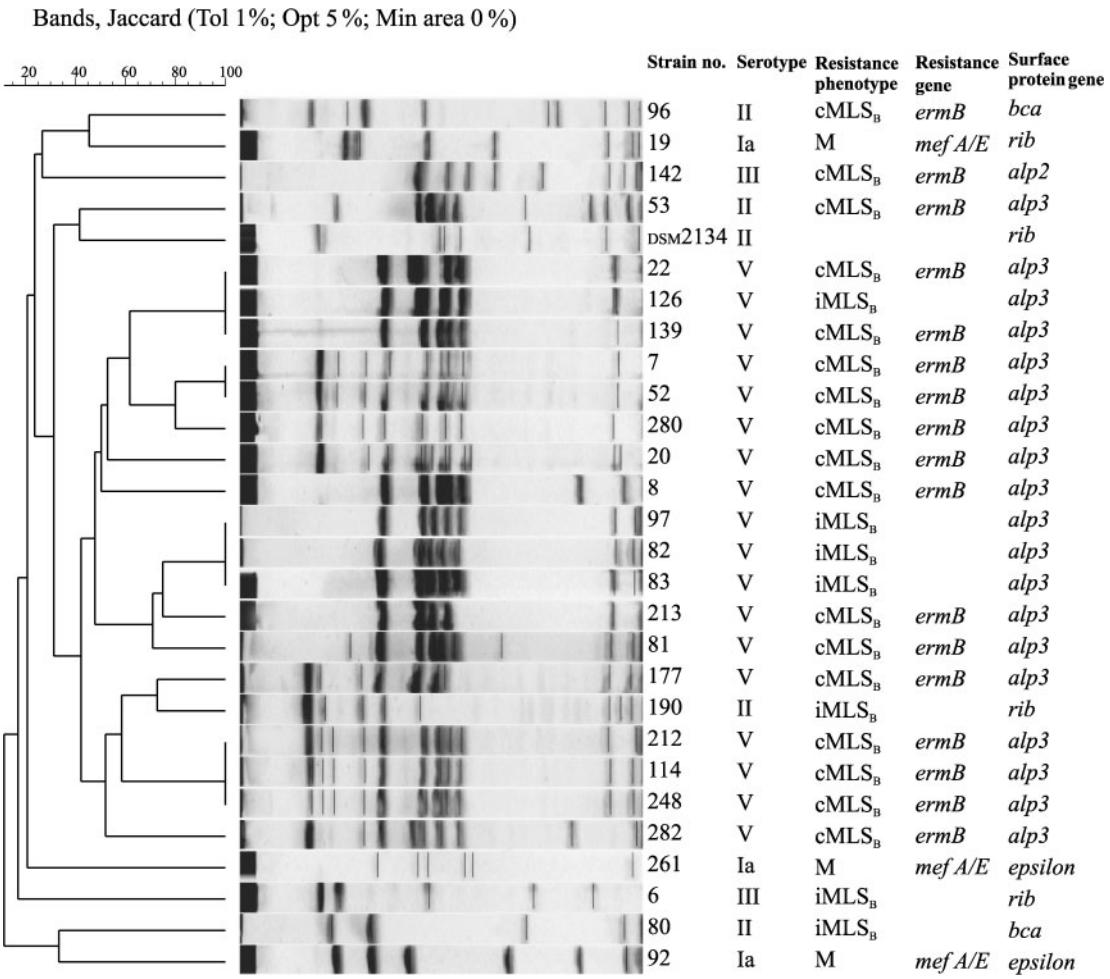

Fig. 4. Cluster analyses of PFGE patterns obtained with *Sma*I-digested chromosomal DNA from 27 *S. agalactiae* isolates with the macrolide-resistance phenotype.

Table 2. Genetic diversity in 27 GBS strains resistant to macrolides grouped according to CPS, resistance phenotypes, resistance genes and genes encoding surface proteins

Investigated feature	No. of GBS isolates	No. of PFGE patterns
CPS type		
Ia	3	3
II	4	4
III	2	2
V	18	11
Total	27	20
Resistance phenotypes		
$cMLS_B$	17	13
$iMLS_B$	7	5
M	3	3
Total	27	21
Resistance genes		
ermB	17	13
mefA/mefE	3	3
Total	20	16
Surface proteins genes		
alp3	19	12
rib	3	3
bca	2	2
epsilon	2	2
apl2	1	1
Total	27	20

precise tool for analysing the molecular epidemiology of GBS.

The Gherardi *et al.* (2007) study conducted with PFGE shows a high genetic diversity among GBS strains that are not macrolide resistant. However, they showed an exact correlation between strains belonging to specific pulsotypes and serotypes, the presence of capsular proteins and genes encoding resistance to macrolides. They also found that in the group of GBS strains resistant to erythromycin, isolated from infections, 60 % of the isolates represented one genetic profile (Gherardi *et al.*, 2007). Similar results were shown by Diekema *et al.* (2003), where GBS isolates resistant to macrolides and belonging to serotype V, collected from blood infections in newborns, in 35 % of cases were of one subtype. According to researchers, the observed phenomenon points to the fact that within the GBS group resistant to macrolides, a clonal spread has occurred in the population prone to the described types of events (Diekema *et al.*, 2003; Gherardi *et al.*, 2007; Schoening *et al.*, 2005).

Analysing the outcomes of our own studies with PFGE, a high genetic diversity was shown between GBS strains resistant to macrolides. Among the analysed features, the highest genetic homogeneity was in the group of isolates representing serotype V (Table 2), which is in agreement with outcomes in the literature (Diekema *et al.*, 2003; Gherardi *et al.*, 2007). It is important to notice that the examined isolates originated from cases of asymptomatic carriage in pregnant women, which could explain the relatively high genetic diversity between strains.

Based on the obtained outcomes, a hypothesis can be made that the observed phenomenon of increasing resistance among GBS isolates originating from colonization not only is the result of clonal spread in the population but also points to horizontal resistance gene transmission between streptococci.

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