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Antimicrobial resistance and molecular characteristics of *Streptococcus agalactiae* isolated from women of reproductive age

Lekooporność i molekularna charakterystyka *Streptococcus agalactiae* izolowanych od kobiet w wieku rozrodczym

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ABSTRACT

Introduction. *Streptococcus agalactiae* infections are among the most significant causes of neonatal invasive diseases. Proper screening and detection of pregnant women carrying GBS allows intrapartum administration of antibiotic prophylaxis and is an effective measure in preventing transmission of bacteria from mother to newborns.

Material and methods. Sixty three bacterial strains were isolated from vaginal swabs from pregnant and non-pregnant women of reproductive age. Species were identified by colony morphology, haemolysis type, Gram staining and SLIDEX® Strepto Plus latex test. Antimicrobial resistance of 56 strains was determined using disk-diffusion method. The presence of molecular resistance determinants was assessed using PCR with specific primers, and capsular types were identified using multiplex PCR.

Results. None of the strains were resistant to the first drug of choice, penicillin. A large percentage of isolates (78.6%) were resistant to doxycycline. The prevalence of resistance to macrolides and lincosamides, antibiotics used in women allergic to penicillin, was high. Those results corresponded with PCR tests, as *tetM* and *ermA1* were most frequently detected genes (98.4 and 87.3%, respectively). 7.94% of strains possessed 7 different out of 13 tested genes determining resistance to different groups of antimicrobials. Among the capsular types, Ia, which proved to be associated with the most severe and invasive infections in mothers and neonates, was the most prevalent (65.08%).

Conclusions. Even though they are susceptible to penicillin, multidrug resistance is common among *S. agalactiae* strains isolated from women of reproductive age and this resistance can be caused by more than one gene per single isolate.

Keywords: *Streptococcus agalactiae*, antimicrobial resistance, capsular types, multiplex PCR, pregnancy.

STRESZCZENIE

Wstęp. Infekcje wywołane przez *Streptococcus agalactiae* są jednymi z głównych przyczyn chorób inwazyjnych noworodków. Badania przesiewowe w kierunku nosicielstwa paciorkowców z grupy B (GBS) u ciężarnych umożliwiają zastosowanie śródporodowej profilaktyki antybiotykowej w celu zapobiegania przenoszeniu bakterii z matki na noworodka.

Materiały i metody. Przebadano 63 szczepy bakterii uzyskane poprzez wymazy pochwowe od ciężarnych i nieciężarnych kobiet w wieku rozrodczym. Bakterie zidentyfikowano na podstawie morfologii kolonii, typu hemolizy, barwienia Grama i testu SLIDEX® Strepto Plus. Profil lekooporności 56 szczepów zbadano metodą dyfuzyjno-krążkową. Występowanie genów warunkujących lekooporność oznaczono techniką konwencjonalnego PCR, natomiast metoda multiplex PCR posłużyła do oznaczenia polisacharydów otoczkowych.

Wyniki. Nie stwierdzono oporności na lek pierwszego wyboru, jakim jest penicylina. 78,6% izolatów było opor-

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nych na doksycyklinę. Często także stwierdzano oporność na makrolidy i linkozamidy, które są antybiotykami stosowanymi u pacjentek uczulonych na penicylinę. Wyniki te korespondowały z wynikami testów PCR, gdyż geny *tetM* i *ermA1* były najczęściej stwierdzanymi genetycznymi determinantami lekooporności (odpowiednio u 98,4 i 87,3% szczepów). Aż 7,94% szczepów *S. agalactiae* posiadało 7 spośród 13 testowanych genów warunkujących oporność na różne antybiotyki. Test multiplex PCR wykazał, że najbardziej rozpowszechniony był typ Ia polisacharydów otoczkowych, powiązany z najcięższymi i najpoważniejszymi infekcjami. Został on wykryty u 65,08% szczepów.

Wnioski. Pomimo całkowitej wrażliwości na penicylinę, wielooporność szczepów *S. agalactiae* izolowanych od kobiet w wieku rozrodczym jest powszechna. Oporność na antybiotyki u tych bakterii może być warunkowana przez występowanie więcej niż jeden gen w jednym izolacie.

Słowa kluczowe: *Streptococcus agalactiae*, lekooporność, polisacharydy otoczkowe, multiplex PCR, ciąża.

INTRODUCTION

Although *Streptococcus agalactiae* (group B streptococcus – GBS) belongs to the normal commensal microflora of gastrointestinal and genitourinary tracts, since 1960s these bacteria have been one of the most significant and increasing causes of neonatal invasive diseases [1–3]. This is because newborn children can acquire GBS from their mothers through aspiration of infected amniotic fluid or during their passage through the birth canal [1].

Proper screening and detection of pregnant women carrying GBS allows intrapartum administration of antibiotic prophylaxis and is an effective measure in preventing transmission of bacteria from the mother to the newborns [4]. Penicillin has been established as a first drug of choice for the prophylaxis and treatment of GBS infections, but clindamycin and erythromycin, as well as tetracyclines, such as doxycycline, have been used as alternatives in penicillin-allergic individuals [5]. Even though, despite the common use of β-lactams, the strains of GBS are still susceptible to penicillin, the resistance to the latter antibiotics has been reported in different countries, thus raising concerns about their use in treatment [5–8]. Some of the most frequently detected resistance mechanisms include the presence of *tetM* and *tetO* genes, associated with plasmids and transposons, respectively, and causing the bacterial ribosome protection [9]. Moreover, the increasing frequency of the use of macrolides, lincosamides and streptogramins promotes the occurrence of bacterial resistance to these antibiotics [10]. The post-transcriptional methylation of adenine residues of 23S ribosomal RNA, mediated by *erm* genes, is among the most frequently observed mechanisms of resistance to this group of antibiotics in GBS.

In Poland, the screening for the presence of this pathogen in pregnant women has become obligatory since April 8th 2011, and based on several studies, it is assumed that about 20% of Polish women are colonized by GBS [6, 11, 12]. The studies have shown that the *S. agalactiae* infections occur in 2-4 out of 1000 newborns, but what is worrying is that the mortality rate of infected newborns reaches up to 20% [6].

One of the most important factors involved in the virulence of *S. agalactiae* includes their capsular polysaccharide (cps), of which there are 10 known to date [13]. Different capsular types are associated with various severity of the eventual infection, as well as there are geographical variations among the prevalence of particular capsular types [13–15].

The aim of this study was to assess the prevalence of *Streptococcus agalactiae* in women of reproductive age in Kraków and to analyze the antibiotic resistance of the selected strains and the presence of genes determining the resistance to most commonly used antibiotics. Finally, the multiplex PCR was employed to classify the isolated strains into different serotype groups. The results obtained in this study will allow for evaluation of the health risk both in women and in newborns. Moreover, they will allow assessment of the treatment efficiency by using the most commonly used drugs of choice and help establish which of the isolated strains are most dangerous to women of reproductive age.

MATERIAL AND METHODS

Microorganisms

A total of 63 GBS isolates recovered from vaginal swabs of pregnant (n=16) and non-pregnant (n=47) women of reproductive age (20–44 years old), patients of the Centre for Microbiological Research and Autovaccines in Kraków, were used in this study. All streptococci were identified to the species level based on the phenotypic methods including colony morphology, type of haemolysis on blood agar, Gram staining and latex test using SLIDEX® Strepto Plus (BioMérieux).

Antimicrobial susceptibility pattern

Antimicrobial susceptibility was tested on 56 strains by the disk-diffusion method on Mueller-Hinton agar (Biocorp) supplemented with 5% sheep blood, using suspensions of 0.5 MacFarland made from fresh bacterial cultures. The isolates were tested for susceptibility to five drugs of choice used for the treatment of GBS, i.e. penicillin, doxycycline, erytromycin, clindamycin and cotrimoxasole. The tests were performed according to the recommendations of the National Reference Centre for Antimicrobial Susceptibility Testing [16]. After incubation, the results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing [17].

DNA extraction

A single bacterial colony was added to 5 ml of Trypticase Soy Broth and incubated overnight at 37°C. The cultures were then centrifuged at 12,200 g for 3 min and the bacterial pellets were resuspended in 100 µl of 10 mM Tris. Total DNA of all 63 GBS isolates was extracted using the Genomic Mini DNA extraction kit (A&A Biotechnology) according to the manufacturer's instructions.

PCR determination of antibiotic resistance genes

Total DNA of the 63 GBS isolates was used to amplify the genes encoding various resistance mechanisms for MLS antibiotics (macrolide, lincosamide and streptogramins) and tetracyclines. The presence of the following genes was examined: *ermA*, *ermA1*, *ermB*, *ermB1*, *ermC*, *int-Tn*, *lnuA*, *vga*, *mefA/E*, *msrA*, *msrA1*, *tetM and tetO* [18–21].

PCRs were performed in a T100 thermal cycler (BioRad). The reactions in a final volume of 25 µl contained 50 ng of DNA template, 12.5 pM of each primer, 2.5 mM of dNTP, 1×PCR buffer and 1 U DreamTaq Green DNA polymerase (ThermoScientific, US). The temperature profile of the reactions consisted of initial denaturation at 95° for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, 45 s of annealing temperature that varied depending on the primers used, 1 min of elongation at 72°C with final extension for 10 min at 72°C. The amplicons were subjected to electrophoresis on 1% agarose (Prona) gels in 1×Tris-Borate-EDTA buffer at 120V for 60 minutes. Agarose gels were stained with SimplySafe (EurX) and photographed using a UV-light gel documentation system GelDoc (BioRad).

Multiplex PCR test for capsular type identification

Multiplex PCR was performed in a T100 thermal cycler (BioRad) and the reaction was conducted in a final volume of 25 µl according to Imperi et al. with 0.3U of Dream Taq Green DNA polymerase (ThermoScientific, US) [13]. Nineteen primers for different capsular types were used in a reaction [13]. The PCR products were subjected to electrophoresis on 2% agarose (PRONA) gels in 1×Tris-Borate-EDTA buffer at 120V for 60 minutes. Agarose gels were stained with SimplySafe (EurX) and photographed using a UV-light gel documentation system GelDoc (BioRad).

Statistical analysis

In order to verify the significance of differences between the antimicrobial resistance, as well as the occurrence of different capsular serotypes in pregnant and non-pregnant women, a χ^2 test was conducted using a Social Science Statistics calculator [22].

RESULTS

Bacterial isolates distribution and antimicrobial resistance

Among 63 isolates, 16 originated from pregnant women and 47 from non-pregnant women. Antimicrobial resistance was determined for 56 strains (10 from pregnant and 46 from non-pregnant women). All of the tested isolates were susceptible to the first drug of choice, which is penicillin. Only 12 isolates (21.4%) were susceptible to doxycyclin, and the following percentages of isolates: 66.1%, 73.2% and 98.2% were susceptible to erythromycin, clindamycin and cotrimoxasole, respectively. There were significant differences among the distribution of susceptible and resistant isolates among pregnant and non-pregnant women with the exception for penicillin (Table I).

Table I. Susceptibility of the tested S. agalactiae isolates to commonly used antibiotics in pregnant and non-pregnant women (%).

Tabela I. Wrażliwość badanych izolatów S. agalactiae na antybiotyki powszechnie stosowane u kobiet ciężarnych i niecięża rnych (%).

Antibiotic	Pregnant (n=10)		Non-pregnant (n=46)	
	S	R	S	R
Penicillin	100	0	100	0
Erythromycin	**80**	**20**	**63**	**37**
Clindamycin	**90**	**10**	**69.6**	**30.4**
Cotrimoxasole	**100**	**0**	**97.8**	**2.2**
Doxycyclin	**50**	**50**	**15.2**	**84.8**

* bolded values indicate significant differences, with $p<0.05$

None of the tested isolates was resistant to all 5 antibiotics. Similarly, none of them was resistant to 4 drugs, but 26.79%, 1.79% and 55.36% of GBS strains were resistant to 3, 2 and 1 antibiotic, respectively. Among the tested isolates only 14.29% were susceptible to all tested antimicrobials.

PCR amplification of antibiotic resistance genes

Table II shows the frequency of detection of individual genes responsible for the resistance to antibiotics of MLS group. Genes *ermA* (645 bp), *ermC* (642 bp), *lnuA* (323 bp) and *vga* (470 bp) were not detected in any of the tested GBS. On the other hand, *ermA1* occurred very frequently, i.e. in 87.3% of isolates. What is interesting, almost all isolates possessed the *tetM* gene. Statistical analysis showed that the differences in the abundance of antimicrobial resistance genes in pregnant and non-pregnant women were statistically insignificant for the MLS group of antibiotics, while for the tetracyclines the difference was significant only in the case of *int-Tn* gene ($p<0.05$), as this gene was observed in 43.75% of isolates from pregnant and in 74.47% of isolates from non-pregnant women.

Among the tested isolates, 7.94% possessed as many as 7 different antimicrobial resistance genes, while in 14.29%, 28.57%, 26.98%, 12.70% and 9.52% 6, 5, 4, 3 and 2 genes were detected, respectively, and this was the smallest number of the detected resistance genes, which means that there were no strains that would not carry any of the examined genes.

Table II. Share of isolates with genes responsible for the resistance to antibiotics belonging to the group of macrolides, lincosamides, streptogramins (MLS) and tetracyclines (%).

Tabela II. Udział izolatów, u których stwierdzono geny odpowiedzialne za oporność na makrolidy, linkozamidy i streptograminy (antybiotyki z grupy MLS) oraz tetracykliny (%).

Gene	No. of strains	%	Product length (bp)
ermA	0	0	645
ermA1	55	87.3	421
ermB	30	47.6	639
ermB1	30	47.6	359
ermC	0	0	642
lnuA	0	0	323
msrA	1	1.6	940
msrA1	20	31.7	400
vga	0	0	470
mefA/E	6	9.5	346
tetM	62	98.4	359
tetO	38	60.3	538
int-Tn	46	73.0	528

Multiplex PCR determination of capsular types

The multiplexed PCR allowed for differentiation of the bacterial isolates depending on the capsular type. Among 10 known serotypes, five were detected in this study, namely Ia, Ib, III, IV and IX (Table III). Capsular type Ia, which was the most prevalent one, occurred in both pregnant and non-pregnant women. The second most frequently detected capsular type was III, which was observed in more than 25% of isolates. Statistical analysis revealed that the differences in the prevalence of GBS capsular types between pregnant and non-pregnant women are statistically significant only in the case of those most frequent types, i.e. Ia and III (Table III).

Table III. Share of isolates with different capsular types from pregnant and non-pregnant women (%).

Tabela III. Udział izolatów z poszczególnymi typami polisacharydów otoczkowych u kobiet ciężarnych i niecięża rnych (%).

Capsular type	Ia (%)	Ib (%)	III (%)	IV (%)	IX (%)
Pregnant	**15 (93.75)**	0	**1 (6.25)**	0	0
Non-pregnant	**26 (55.32)**	3 (6.38)	**15 (31.91)**	2 (4.26)	1 (2.13)
Total	41 (65.08)	3 (4.76)	16 (25.40)	2 (3.17)	1 (1.59)

*bolded numbers indicate significant differences at $p<0.05$

DISCUSSION

Among 63 strains, 16 were isolated from pregnant women. The detection of *S. agalactiae* carriage in pregnant women allows for the administration of proper intrapartum prophylaxis in the form of antibiotics, i.e. penicillin or, in the case of penicillin-allergic patients, other drugs of choice [1]. Correctly administered antibiotic treatment prevents transmission of bacteria from the mother to the newborn; however, children born by women in which intrapartum antibiotic prophylaxis was administered, still need to be subjected to at least 24-hour observation [6, 23].

Antimicrobial resistance, spreading through various pathways among *S. agalactiae*, currently seems to be the most important problem [24]. Our study showed that the number of resistant strains increases, resulting in multidrug resistance among bacteria. The highest resistance rate recorded in this study involved tetracyclinces, since as much as 78.6% of examined isolates were resistant to doxycyclin. Also high resistance rate was recorded in the case of macrolides and lincosamides, i.e. 33.9 and 26.8% to erythromycin and clindamycin, respectively, with

5.36% of strains resistant only to erythromycin. Even though some authors report similar rate of resistance to these groups of antibiotics, the recorded rate is disturbingly high, as both of these antibiotics are the drugs of choice in the case of women allergic to penicillin [25, 26, 27]. Moreover, these results indicate that the prevalence of resistance to macrolides and lincosamids among GBS has increased, since resistance recorded by Brzychczy-Włoch in the study conducted in 2010 in one of the hospitals in Kraków demonstrated 16% and 10% of strains resistant to erythromycin and clindamycin, respectively [28]. On the other hand, Pruss et al. in their study conducted in the period of 2010-2013 in the West Pomeranian region reported MLSB phenotype in 28% of strains and MSB phenotype (resistance only to erythromycin) in 2% of strains [29]. Prośniewska et al. in their study conducted in 2010-2012 in Łódź observed that 45.45% of vaginal GBS strains were resistant to erythromycin and 34.09% to clindamycin. However, an important observation is that all of the examined isolates were susceptible to penicillin, which is the first drug of choice in the prophylaxis of GBS infections [23].

The conducted molecular analyses showed that the most prevalent genes responsible for the MLS resistance included *erm* genes, with *ermA1* being most frequently detected, i.e. among 87.3% of isolates and almost 50% of *ermB* (Table II). Both *ermA1* and *ermB1* genes are responsible for dimethylation of the adenine residue in 23S rRNA, resulting in ribosomal changes and therefore reduced affinity between ribosomes and MLS antibiotics [31]. According to Poyart et al., the presence of *ermB* genes is more frequent in multidrug resistant strains, which was also detected in our study [32]. Another gene, quite frequently detected in this study, i.e. *msrA1* encoding the efflux pump, which was observed in 31.7% of strains in our research, has not been reported with such frequency so far [31, 33]. The results of molecular analysis demonstrated also the high prevalence of genes responsible for resistance to tetracyclines, as *tetM* was observed in as much as 98.4% of isolates. This was also confirmed by the phenotypic tests, in which resistance to doxycyclin, which belongs to the group of tetracyclin antibiotics, was the most frequently observed. This gene encodes a ribosome protection mechanism and, similarly, high prevalence of this gene in GBS was observed by other authors [34, 35].

A multiplex PCR was employed to assess the capsular type of the examined GBS strains, which proved to be a useful, efficient, reliable and rapid method in identification of this important virulence factor of *S. agalactiae* [13]. This is an important factor, as the capsular polysaccharide has been recognized as one of the most important virulence factors [36]. It has been reported by different authors that predominating serotypes change over time and vary by geographical region. Moreover, the coexistence of several serotypes constitutes a major obstacle in the development of a global and effective vaccine against GBS to prevent neonatal infections [37]. The study revealed the presence of five among the 10 already known capsular types. In this study, serotype Ia was the most frequently detected (65.08% of total isolates), followed by serotype III (25.40%). These results are in accordance with those obtained by Oviedo et al., where serotypes... Ib, IV and IX were also detected, but in much smaller numbers [1]. These results are consistent with observations of other authors, who reported serotype Ia to be the most prevalent not only in different European countries but also in US and South America [1, 36, 38]. The highest prevalence of serotype Ia coupled with increased resistance to certain antibiotics may raise concerns as this serotype proved to be associated with the most severe and invasive infections both in mothers and neonates [39]. The present study showed that the resistance to both erythromycin and clindamycin was observed in GBS isolates of capsular types Ia, Ib and III, which differs from the results of Otaguiri et al., who observed the resistance to these both antibiotics only among isolates belonging to capsular types III and V [5]. On the other hand, as in Otaguiri et al., isolates of exclusively Ia capsular types showed resistance only to erythromycin [5].

CONCLUSIONS

This study revealed that the resistance to tetracyclinces is one of the most common mechanisms of resistance among the isolated GBS and only 14.29% were susceptible to all of the tested antibiotics. Also high prevalence of strains resistant to clindamycin and erythromycin indicates that the administration of these drugs to penicillin-allergic women should be preceded by an adequate antimicrobial susceptibility testing. However, penicillin, the first drug of choice used as a means of intrapartum prophylaxis of GBS infections, proved to be the most effective, as all examined strains were susceptible.

The experiments showed that the multidrug resistance is common among *Streptococcus agalactiae* strains isolated from women of reproductive age and that the resistance to antibiotics can be caused by

the presence of more than one gene per single isolate. Capsular type Ia, which is proved to cause ones of the most invasive GBS infections, was the most frequently found in both pregnant and non-pregnant women.

Due to raising frequency of *Streptococcus agalactie* carriage among women, there is an increasing risk of transmission of this bacterium to newborns, which is associated with significant threat to their health and life. Some of the antimicrobial resistance detected in this study can be disturbing; however, a proper identification and selection of antibiotics, in the form of antibiogram, should be sufficient to eliminate bacteria from the body of both mother and child.

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