Dynamics of colonization with group B streptococci in relation to normal flora in women during subsequent trimesters of pregnancy

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SUMMARY

The main objective of the study was to compare the qualitative and quantitative composition of vaginal and rectal flora in GBS-positive (n=15) and GBS-negative (n=27) pregnant women examined in three subsequent trimesters of their pregnancy. Study samples consisted of vaginal and rectal smears and urine samples. GBS numbers were determined by the quantitatively cultured method [cfu/ml] and with the use of qPCR. Five GBS colonies were isolated per each positive sample and genotyped by PFGE and serotyping. The normal flora components: *Lactobacillus*, *Bifidobacterium* and *Candida* were quantitatively cultured. Carriage of GBS in subsequent trimesters in vagina/anus was variable and fluctuated between 17% and 28%. Quantitative GBS analyses showed that the vaginal population was at a constant level with the mean value equal to 3.94×10^4 cfu/ml, in contrast to the rectal population where the highest values appeared in the third trimester 4.37×10^5. The use of qPCR gave 7% more positive results for vaginal/rectal swabs. Genetic similarity analysis showed that one GBS clone was present in 73% of carriers during pregnancy, while in 27% of patients, 2 clones were found. H_2O_2-positive vaginal lactobacilli were detected in all women, while H_2O_2-negative lactobacilli and *Bifidobacterium* occurred more frequently in the anus in about 50% of women. Candida was present in the vagina in 30% of women. The analysis of women in three consecutive trimesters of pregnancy on the basis of a study group and control group showed no statistically significant differences in either the species (qualitative) or quantitative composition in vaginal and rectal flora in both of the groups. Therefore, GBS should be considered as a component of the microbiota and an opportunistic microorganism rather than a typical pathogen, because it does not distort the composition of women's normal genital tract flora.

KEY WORDS: Group B streptococcus (GBS), Lactobacilli, Pregnant women, Colonization dynamics.

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INTRODUCTION

The bacterial flora of the female reproductive tract make up complex ecosystem consisting mostly of lactic acid bacteria (LAB), represented by the Lactobacillus species, but also potentially pathogenic microbes (Bayó *et al.*, 2002). A special threat among the microbes colonizing the vagina is posed by the beta-hemolytic Group B Streptococci (GBS) represented by *Streptococcus agalactiae*, which colonizes mainly the epithelial cells of the lower gastrointestinal tract and the vagina. GBS carriage in pregnant women's reproductive tracts reaches 10-40% of subjects and as many as 40% develop so-

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called asymptomatic bacteriuria. The digestive tract is considered to be the main reservoir of *S. agalactiae*, which most probably constitutes the source of vaginal and urinary colonization (Schrag *et al.,* 2002). Epidemiological data confirm the relation between the rectal and vaginal colonization of *S. agalactiae* in pregnant women, and infant infections triggered by this bacterium. During delivery, there is a danger of infant colonization with GBS coming from the mother's microflora of the vagina or cervical canal through vertical transmission. Group B streptococci colonize the infant's oral cavity and subsequently the respiratory and gastrointestinal tracts. The risk of GBS transmission equals 70%. GBS may trigger infant infections which can be divided, depending on time of occurrence after delivery, into early onset diseases manifesting mainly in the form of pneumonia, and late onset diseases most frequently emerging in the form of meningitis and sepsis (Melin *et al.,* 2011).

Screening of pregnant women for GBS carriage in the third trimester, between the 35th and 37th week of gestation, constitutes recommended infection prophylaxis in newborns. It is advised to collect samples from the vagina and rectum simultaneously, conduct their preliminary incubation on a selective growth medium and, in the event of obtaining a positive result, perinatal antibiotic prophylaxis (Schrag *et al.,* 2002; Verani *et al.,* 2010).

S. agalactiae is a genetically diverse organism. Therefore, a combination of several molecular typing methods, such as serotyping or PFGE profiling should be considered to gain a better understanding of the pathogenesis and epidemiology of GBS isolates (Perez-Ruiz *et al.,* 2004; Poyart *et al.,* 2007). GBS strains are subdivided according to their type-specific capsular polysaccharides (CPS) into ten unique serotypes (Ia, Ib, II - IX). GBS pathogenicity varies between serotypes: in particular, the serotypes most often causing invasive infections in newborns are III, Ia and V (Poyart *et al.,* 2007).

To our knowledge, only two studies (Hansen *et al.,* 2004; Perez-Ruiz *et al.,* 2004) have addressed PFGE typing and serotyping of *S. agalactiae* within individual women, another one addressed RAPD (El Aila *et al.,* 2009a), and one - MLST (Manning *et al.,* 2008). Similarly, there is very little work on the changes in the normal flora of the vagina and anus during pregnancy (Gustafsson *et al.,* 2011; Petricevic *et al.,* 2012). Moreover, little is known about the regulation of the GBS population in the genitourinary tract and rectum by other components of normal flora. Therefore, the main objective of this study was to monitor the dynamics of the *Streptococcus agalactaie* population and to compare the qualitative and quantitative composition of the vaginal and rectal flora in GBS-positive and GBS-negative pregnant women examined in three subsequent trimesters of their pregnancies.

MATERIALS AND METHODS

Study population

The study included 42 healthy pregnant women aged 22-35 years old, without clinical signs of genitourinary infections, with values of 1-6 according to Nugent score (Nugent *et al.,* 1991).

Inclusion criteria: women between the ages of 18 and 40; pregnant women in the first trimester of gestation (1st-13th week of gestation); without clinical symptoms of genitourinary infections requiring antibiotherapy; confirmed physiological composition of genitourinary tract flora according to the 10-point Nugent score (results: 0-6 value); written permission to take part in the study.

Exclusion criteria: women under 18 and over 40; pregnant women with the so-called high-risk pregnancy; rupture of membranes; gestational diabetes; antibiotherapy in the period of up to 30 days before getting pregnant or during gestation; diagnosis of bacterial vaginosis diagnosed on the basis of a direct smear from a vaginal swab stained by Gram's method (results 7-10 points in the 10-point Nugent score) and on the basis of culture; clinical symptoms of genitourinary infection requiring antibiotherapy; lack of written permission to take part in the study.

The study group consisted of 15 women who had confirmed *S. agalactiae* colonization of the genitourinary tract and/or anus at least once during the pregnancy. The control group consisted of 27 women without *S. agalactiae* colonization in the study pregnancy. On the basis of

the collected questionnaires, a characteristic of the studied group of women was devised, with special attention paid to patients colonized with GBS (Table 1). The following features were analysed: age, pH, GBS carriage in the previous pregnancy, miscarriages for various reasons, vaginal inflammation up to 12 months before pregnancy, inflammation of the urinary tract up to 12 months before pregnancy, chronic diseases, smoking, occupation, place of residence, sexual activity, hormonal contraception used before pregnancy, past surgeries performed on ovaries and/or genitals and past radiotherapy or chemotherapy. There were no statistically significant differences between the studied groups of women.

Specimen collection

The screening for GBS carriage was performed in three trimesters of pregnancy. The materi-

TABLE 1 - *Characteristics of the studied group of women (n=42), divided into patients colonized with group B streptococci (n=15) and uncolonized women (n=27).*

Studied features	*GBS-positive women (n=15)*	*GBS-negative women (n=27)*	*All women studied (n=42)*
Average age [years]	30.6	30.7	30.6
Average pH	4.7	4.8	4.8
GBS carriage in previous pregnancy [number/total (%)]	2/15 (13%)	3/27 (11%)	5/42 (12%)
Women who miscarried for various reasons [number/total (%)]	4/15 (27%)	6/27 (22%)	10/42 (24%)
Vaginal inflammation up to 12 months before pregnancy [number/total (%)]	5/15 (33%)	8/27 (29%)	13/42 (31%)
Inflammation of the urinary tract up to 12 months before pregnancy [number/total (%)]	1/15 (7%)	6/27 (22%)	7/42 (17%)
Chronic diseases [number/total (%)]	1/15 (7%)	4/27 (15%)	5/42 (12%)
Smoking [number/total (%)]	1/15 (7%)	1/27 (4%)	2/42 (5%)
Occupation [number/total (%)]			
1. white-collar worker	8/15 (53%)	23/27 (86%)	31/42 (74%)
2. blue-collar worker	3/15 (20%)	3/27 (11%)	6/42 (14%)
3. unemployed	4/15 (27%)	1/27 (3%)	5/42 (12%)
Place of residence [number/total (%)]			
city >1mln	8/15 (53%)	12/27 (45%)	20/42 (48%)
town <1mln	4/15 (27%)	6/27 (22%)	10/42 (24%)
village	3/15 (20%)	9/27 (33%)	12/42 (28%)
Sexual activity [number/total (%)]			
<4x/month	3/15 (20%)	5/27 (18%)	8/42 (19%)
>5-10x/month	8/15 (53%)	12/27 (45%)	20/42 (48%)
>11x/month	4/15 (27%)	10/27 (37%)	14/42 (33%)
Hormonal contraception used before pregnancy [number/total (%)]	9/15 (60%)	17/27 (63%)	26/42 (62%)
Past surgeries performed on ovaries and/or genitals [number/total (%)]	2/15 (13%)	5/27 (18%)	7/42 (17%)
Past radiotherapy or chemotherapy [number/total (%)]	0/15 (0%)	2/27 (7%)	2/42 (5%)

als taken for the study were constituted by two swabs from posterior vaginal fornix and one swab from anus, as well as a urine sample. In order to standardize the method and to eliminate the variations in the amount of collected material the swabs were previously wetted in 100 µl of 0.9% NaCl. The materials were taken from the patients in the course of a control gynaecological examination in subsequent trimesters of pregnancy and, in a few cases, in the puerperium. Additionally, the pH of vaginal fluid was measured using pH test strips (Sigma). Vaginal and anal smears were placed in a non-nutrient Amies transport medium (Eurotubo) and urine samples in a sterile box, and were cultured within 2–4 h on the chosen medium. The first vaginal smear served to prepare a direct smear stained with the Gram's method, which enables the evaluation of the vaginal flora condition with the use of a 10-point Nugent score (Nugent *et al.,* 1991). The second one was used to assess vaginal flora composition quantitatively, taking into account bacteria from the *S. agalactiae* species and chosen components of genitourinary tract physiological flora such as *Lactobacillus* spp., *Bifidobacterium* spp. and *Candiada* spp. Anal smear was used to determine *S. agalactiae* carriage and chosen components of normal gastrointestinal tract flora, as the above. The urine sample underwent qualitative GBS test.

Quantitative assessment of *S. agalactiae* and chosen components of normal flora in vaginal and anal smears and in urine samples carried out in cultures

In order to obtain the proper volume of starting material, which is subjected to quantitative assessment for chosen microbial groups, the vaginal and anal smears were suspended separately in 1 ml NaCl and vortexed for 1 minute. Subsequently, 100 µl (dilution 10^0) of the starting material was taken from the sample and transferred onto Columbia 5% sheep blood agar (Difco) for GBS culture. The next 100 µl of the starting material were used to prepare a series of dilutions (10^{-1}-10^{-7}) in 0.9 ml 0.9% NaCl, which were subsequently cultured 100 µl per the appropriate medium for chosen microbial groups cultures: Columbia 5% sheep blood agar (Difco) for *S. agalactiae* culture; MRS agar (Oxoid) for bacteria of the species *Lactobacillus* spp.; Bifidobacterium agar (Becton Dickinson) for *Bifidobacterium* spp.; and Sabouraud agar (Oxoid) for yeast-like fungi culture of the species *Candida* spp. The screening method for GBS carriage was carried out according to the Centers for Disease Control and Prevention (CDC) recommendations (Schrag *et al.,* 2002). Therefore, 300 µl of the starting material were suspended in 1 ml Todd–Hewitt broth (Difco) supplemented with 8 µg/ml gentamicin and 15 µg/ml nalidixic acid (Oxoid), then incubated in aerobic conditions overnight at 37°C and after that subcultured onto Columbia 5% sheep blood agar (Difco) for the purpose of *S. agalactiae* culture. As regards the materials cultured on MRS and BL agar, the culture was carried out in anaerobic conditions at 37°C for 48 h, and on Sabouraud medium in aerobic conditions at 37°C for 24-48 h. Microbes belonging to the species *Lactobacillus* spp., *Bifidobacterium* spp. and *Candida* spp., representing physiological flora, were cultured and identified according to standard procedures concerning culture, isolation, and identification of these microbial groups (Sneath *et al.,* 1986). Additionally, the isolated bacterial strains from the species *Lactobacillus* spp. were determined H_20_2-positive and H_20_2-negative, using the Peroxide Test (Merck) (Strus *et al.,* 2006).

A 1 µl urine sample was cultured onto Columbia 5% sheep blood agar (Difco) for quantitative *S. agalactiae* culture and on MacConkey medium (Difco) for rods culture. *S. agalactiae* isolation from urine samples in the amount of $\geq 10^4$ cfu/ml and lack of leukocyturia were adopted as microbiological criteria of confirmed asymptomatic significant bacteriuria (Verani *et al.,* 2010). In total, 390 materials were examined and cultured on 8600 different microbiological media employed in the diagnostics of the studied microbes. Out of the GBS-positive materials (n=56), five randomly selected *S. agalactiae* colonies were isolated from each one, yielding 280 isolates, later secured for further detailed characteristics, such as serotyping and genotyping by PFGE method.

Quantitative real time PCR

In order to assess the studied materials for *S. agalactiae* using the quantitative real time

PCR (qPCR) method, 500 µl of starting material obtained through suspending vaginal and anal smears in 0.9 ml 0.9% NaCl (see above) and 500 µl of urine, the bacterial DNA was isolated with the use of the nucleic acid isolation kit Genomic Mini (DNA Gdansk) according to the manufacturer's protocol. The PCR amplifications were conducted on the DNA isolates to detect the *cfb* gene encoding the CAMP factor present in every GBS (Ke *et al.,* 2000) using the ready-to-use kit JumpStart TaqReadyMix (Sigma). The reactions were carried out in a CFX96 thermocycler (BioRad), and the fluorescence was monitored throughout the reaction. DNA from the reference strain of *S. agalactiae* (DSM 2134) was prepared in serial dilutions from 10^1 to 10^8 cells and was used in a
series of qPCRs. Threshold cycle was the cycle number when the threshold was reached by fluorescence. Standard curve was generated from the amplification plot ($y=-1.413\ln(x) +39.985$; correlation coefficient (R^2) =0.9866). Detection and quantitation were linear over the range of DNA concentrations examined. To determine the number of GBS cells, the fluorescent signals detected from DNA (in duplicate) in the linear range of the assay were averaged and compared to a standard curve.

PFGE and cluster analysis

Two hundred and eighty GBS isolates were typed by pulsed-field gel electrophoresis (PFGE). DNA was digested using *Sma*I (MBI Fermentas) and the fragments separated in the CHEF-DR III device (Bio-Rad), according to the previously described method (Skjaervold *et al.,* 2004).
The genetic similarity between isolates was calculated using Molecular Analyst (Applied Maths) software, making use of the Jaccard coefficient and the unweighted pair group method with arithmetic mean (UPGMA) as a method for clustering with 3% tolerance and 0.5% optimization settings. Genotype patterns were compared according to the guidelines of van Belkum *et al.* (van Belkum *et al.,* 2007).

Serotyping

All genetically different PFGE types of GBS isolates were analysed. The serotype characteristics were tested using serological Group B Streptococcus GBS Serotyping Kit (ESSUM, Sweden). To verify the method, the detection of genes encoding capsular polysaccharides Ia, Ib, II-VIII was investigated using multiplex PCR method with specific primers (Genomed) according to Poyart *et al.* (Poyart *et al.,* 2007). For the purpose of DNA isolation, the GBS isolates were cultured in the TSB medium (Difco) at the temperature of 37°C for 24 h. The bacterial DNA was isolated with the use of the nucleic acid isolation kit Genomic Mini (DNA Gdansk) pursuant to the manufacturer's protocol. The PCR products were separated after amplification in 2% agarose gel in 0.5×TBE buffer (Fluka) in the presence of ethidium bromide (0.25 µg/mL) (Sigma). The final analysis was conducted with the application of Quantity One software (BioRad) and gel visualization apparatus Gel-Doc2000 (BioRad).

Statistical analysis

For statistical analysis, the Kruskal-Wallis, Wilcoxon, Cochran-Mantel-Haenszel and Pearson's Chi-squared tests were used. The tests were used to compare GBS-positive women and GBS-negative women in the presence of bacteria in vagina and rectum and to examine correlation. *P* values of <0.05 were considered significant. Analyses were conducted using SAS 9.1 package and SAS Enterprise Guide 3.0 (SAS Institute Inc, Cary, NC, USA).

RESULTS

Group B streptococci carriage

The observed colonization of genitourinary tract and/or anus with GBS was continuous, intermittent, or periodic in nature depending on the examined patient. In the studied group of 42 patients, GBS carriage confirmed in at least one trimester rate was 36% (15/42), but when analysing the trimesters separately, different carriage rates could be demonstrated, in the first trimester it was 28% (12/42), in the second trimester 26% (11/42) and in the third trimester 24% (10/42). Carriage of group B streptococcus in vagina in the first trimester was 24%, in the second 18% and in the third 17%; in anus it was 19%, 24% and 17%, respectively (Figure 1).
When analysing the extent of GBS colonization, the number of colony forming units (cfu) per 1

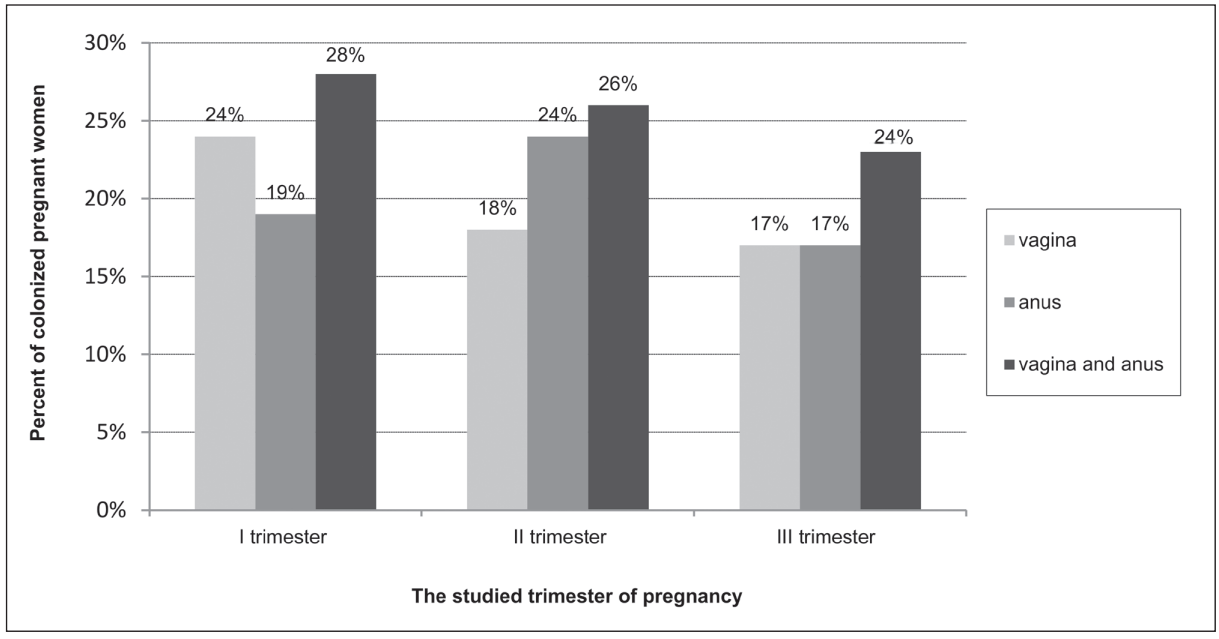

FIGURE 1 - *Carriage of group B streptococcus (GBS) in subsequent trimesters of pregnancy.*

ml was determined. The highest values reached $1x10^6$ cfu/ml for vaginal smears, $4x10^6$ cfu/ml for anal smears and $4x10^5$ cfu/ml for urine samples, whereas the lowest values reached $2x10^1$ cfu/ml for vaginal and anal smears, $1x10^3$ cfu/ml for urine samples (Figure 2).

The monitoring of quantitative results in the group B streptococci population was presented through the calculation of average GBS culture results [cfu/ml] for the vaginal, anal and urine samples in subsequent trimesters of pregnancy (Figure 3).

It was demonstrated that in the course of the three trimesters studied, the GBS population in the vagina did not exceed the standard number and oscillated in the average region of $7.43x10^4$ cfu/ml in the first trimester to $1.74x10^4$ cfu/ml in the third. However, an increase in the number of GBS was observed as regards anal smears, which reached a mean value of $4.37x10^5$ cfu/ml in the third trimester, in relation to $2.8x10^4$ cfu/ml in the first.

Asymptomatic GBS bacteriuria

Asymptomatic GBS bacteriuria ($\geq 1\times10^4$cfu/ml) was shown in 13% (2/15) of GBS carriers. The mean values from urine cultures for three trimesters remained at a constant level from 1.13×10^4 in the first trimester to 2.65×10^4 cfu/ml in the second (Figure 3). The relation was demonstrated between the occurrence of asymptomatic bacteriuria in patients and massive vaginal and anal colonization with GBS and *S. agalactiae* in a particular patient throughout the study period.

Quantitative assessment of *S. agalactiae* in vaginal and anal smears and in urine samples carried out with the quantitative real time PCR method

In order to determine the threshold value (sensitivity) of the quantitative real time PCR method, the obtained results were compared to the results of cultures. These results for DNA samples isolated from vaginal and anal smears were comparable to the results from cultures. However, in isolated cases, values higher by one or two orders of magnitude were obtained. Furthermore, for 7% of materials originating from the vagina and anus, positive results were obtained with the qPCR method, as against the culture method, in which *S. agalactiae* growth was not observed. Yet, in the case of all GBS-positive urine samples, results from the qPCR method were lower by two orders of magnitude when compared to culture results.

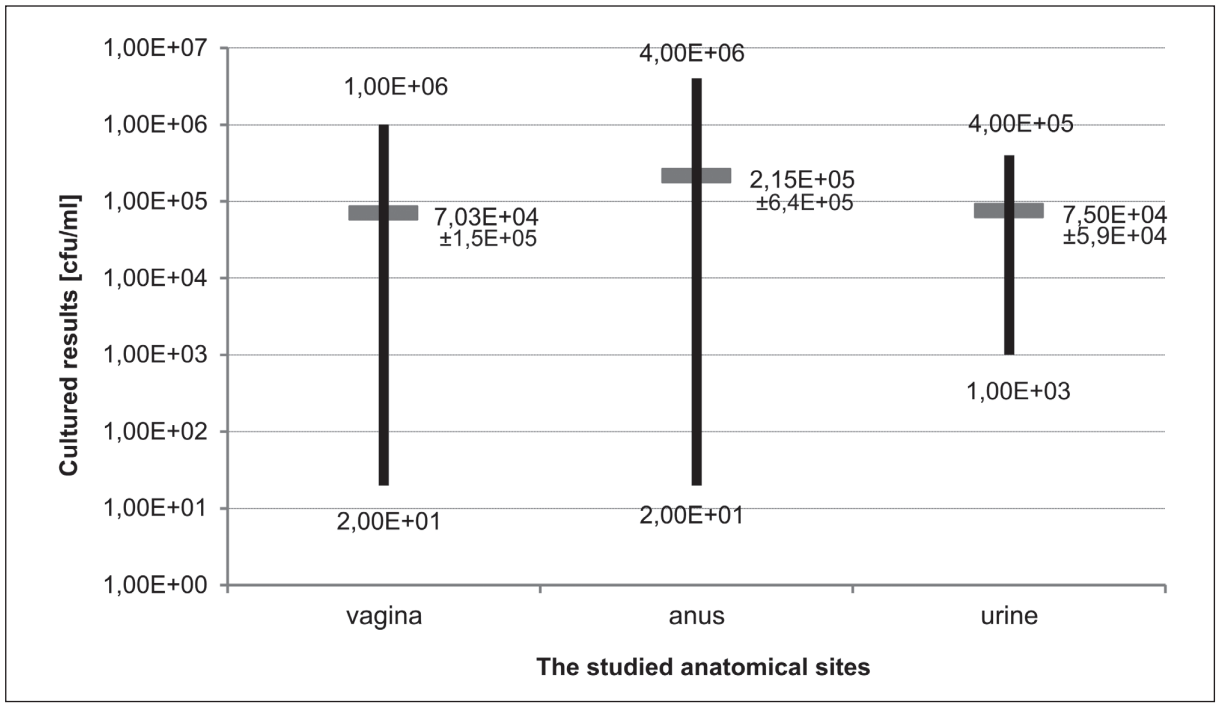

FIGURE 2 - *Average GBS culture results [cfu/ml] with maximum and minimum values in relation to the site of isolation, such as vagina, anus and urine samples analysed in GBS-positive pregnant women during pregnancy.*

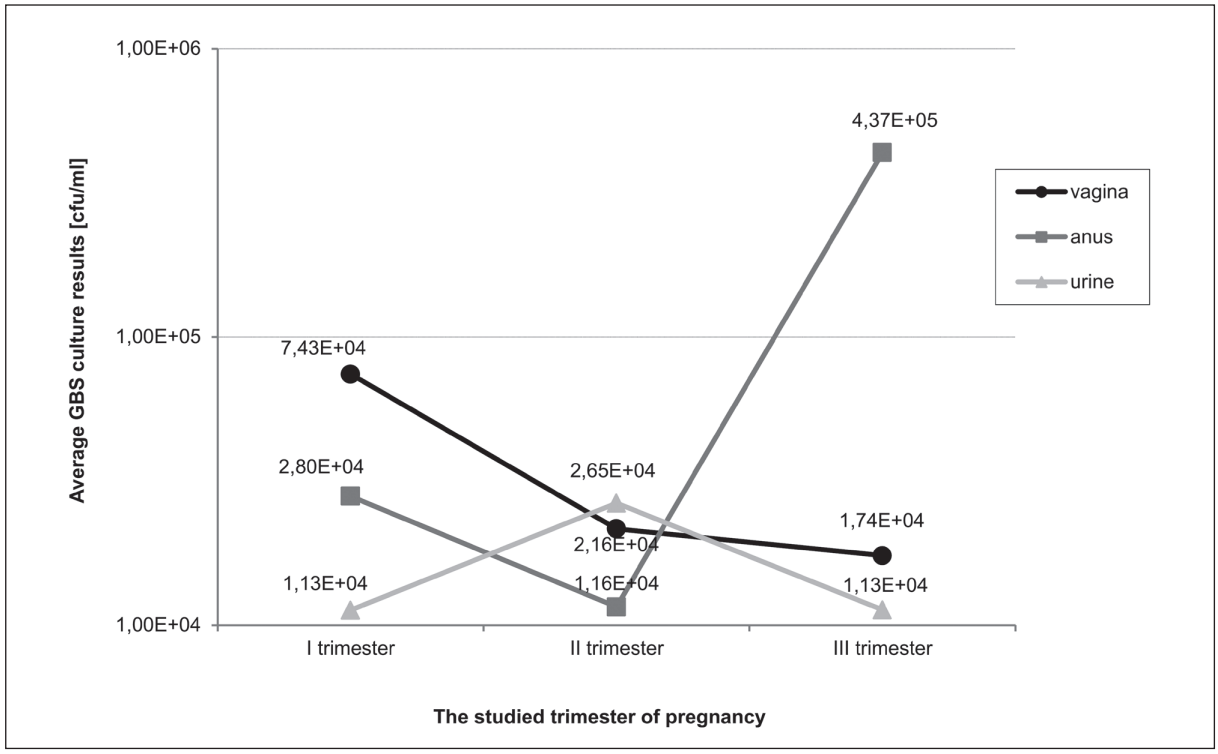

FIGURE 3 - *Average and standard deviations of GBS culture results [cfu/ml] for the vaginal, anal and urine samples in subsequent trimesters of pregnancy in GBS-positive pregnant women.*

Analysis of genetic similarity of GBS isolates by PFGE and serotyping

In order to conduct an analysis of genetic similarity of GBS isolates, their restriction patterns were analysed (Figure 4) and their serotype determined. Thanks to the PFGE method, genetic profiles of GBS isolates originating from carriage were compared, demonstrating the occurrence of two different GBS clones for 27% (4/15) of pregnant women. The clones belonged to different serotypes periodically present in the vagina or anus. With the remaining 73% (11/15) of patients, the presence of only one GBS clone occurring periodically or permanently in the genitourinary tract or anus was confirmed. What is more, it was confirmed that the observed colonization of the genitourinary tract results from the presence of the same GBS clones in different trimesters and in the puerperium.

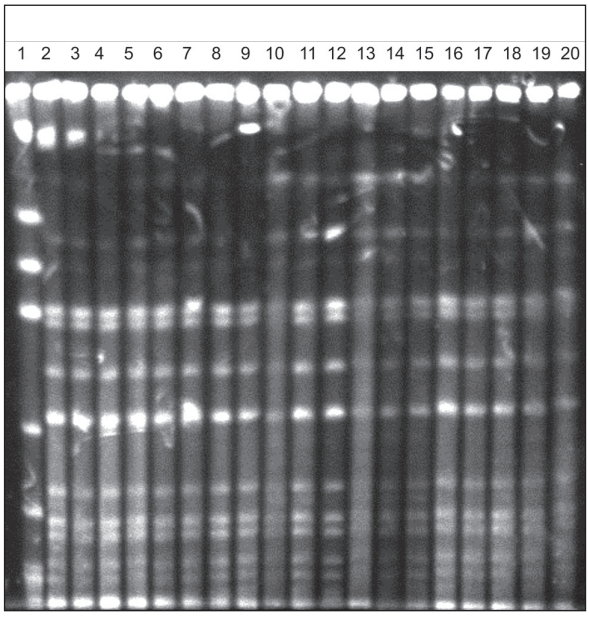

FIGURE 4 - *Genetic profiles of S. agalactiae isolates, originating from vaginal swab, anal swab and urine sample from patient number 2, subjected to DNA digestion by restriction enzyme SmaI, carried out with the pulsed field gel electrophoresis (PFGE) method. Legend: 1: the reference strain of S. agalactiae DSM 2134; 2-7: S. agalactiae strains isolated in I trimester from: 2,3: vagina; 4,5: anus; 6,7: urine; 8-13: S. agalactiae strains isolated in II trimester from: 8,9: vagina; 10,11: anus; 12,13: urine; 14-20: S. agalactiae strains isolated in III trimester from: 14,15: vagina; 16,17: anus; 18,19: urine; 20: S. agalactiae strains isolated in the puerperium from a vaginal smear.*

Analysis of changes in the GBS population in relation to the composition of physiological vaginal flora

Analysis of changes in the GBS population in relation to selected components of the normal flora in vagina and anus in the subsequent trimesters of pregnancy is presented in Figure 5. Based on the classical culture method, lactobacilli were found and isolated from all pregnant women (n=42) but the rate was variable depending on the trimester (Figure 5). We demonstrated that 100% of pregnant women (n=42) in the third trimester had H_2O_2-positive lactobacilli in the vagina and 41% (n=17) in rectal smears, moreover, 27% (n=11) of pregnant women had H_2O_2-negative lactobacilli in the vagina and 55% (n=23) in the anus. *Bifidobacterium* spp. occurred in anal smears in 38% (n=16) to 53% (n=22) of pregnant women, but it was almost absent from the vagina. The presence of *Candida* spp. in the vagina remained stable at around 30% (n=13) in subsequent trimesters of pregnancy, but in the rectum it reached the level of 24% (n=10) in the first trimester, and 10% (n=4) in the third.

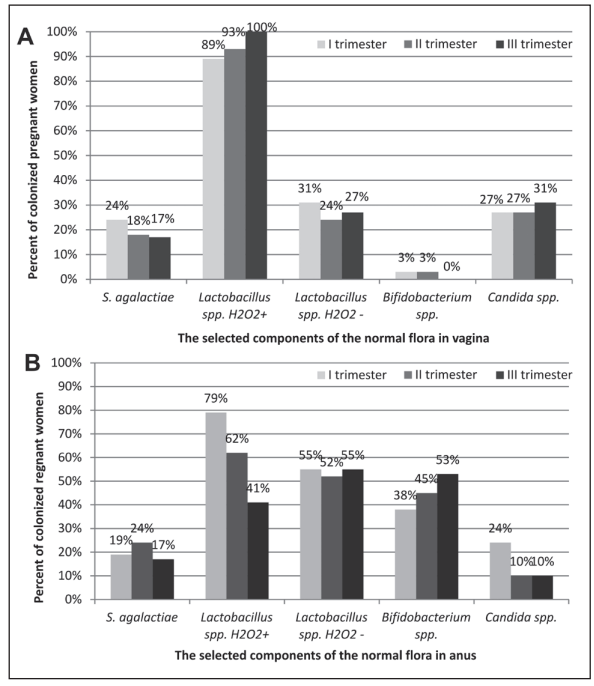

FIGURE 5 - *Analysis of changes in the GBS population in relation to selected components of the normal flora in vagina (A) and anus (B) in the women studied in subsequent trimesters of pregnancy.*

TABLE 2 - *Average bacterial number and standard deviations of culture results [cfu/ml] of different species of bacteria isolated from vaginal and anal smears collected from patients colonized with group B streptococci (GBS+) and uncolonized women (GBS-).*

	I trimester			*II trimester*			*III trimester*		
	Average bacterial no. ± SD [cfu/ml]			*Average bacterial no. ± SD [cfu/ml]*			*Average bacterial no. ± SD [cfu/ml]*		
	GBS+	*GBS-*	*Total*	*GBS+*	*GBS-*	*Total*	*GBS+*	*GBS-*	*Total*
S. agalactiae (GBS)									
Vagina	7.43E+04	0.00E+00	2.65E+04	2.16E+04	0.00E+00	7.96E+03	2.20E+04	0.00E+00	7.35E+03
	±2.48E+04	±0.00E+00	±1.53E+05	±7.72E+04	±0.00E+00	±4.80E+04	±4.93E+04	±0.00E+00	±3.03E+04
Ainus	2.80E+04	0.00E+00	1.00E+04	1.25E+04	0.00E+00	4.38E+03	4.37E+05	0.00E+00	1.46E+05
	±6.87E+04	±0.00E+00	±4.32E+04	±2.74E+04	±0.00E+00	±1.73E+04	±1.29E+05	±0.00E+00	±7.72E+05
Urine	1.13E+04	0.00E+00	4.02E+03	2.65E+04	0.00E+00	9.76E+03	1.32E+04	0.00E+00	4.40E+03
	±2.87E+04	±0.00E+00	±1.80E+04	±9.53E+04	±0.00E+00	±5.92E+04	±3.28E+04	±0.00E+00	±1.99E+04
Lactobacillus spp. H_2O_2+									
Vagina	1.57E+06	1.42E+06	1.73E+06	2.61E+06	1.05E+06	1.62E+06	8.15E+05	1.53E+06	1.29E+06
	±2.06E+06	±2.57E+06	±2.40E+06	±5.05E+06	±1.32E+06	±3.33E+06	±8.46E+05	±2.84E+06	±2.40E+06
Ainus	6.29E+04	7.45E+04	8.11E+04	3.74E+04	2.75E+04	3.12E+04	1.44E+05	6.11E+04	8.97E+04
	±1.60E+04	±1.61E+04	±1.61E+04	±7.49E+04	±5.12E+04	±6.12E+04	±2.74E+05	±1.75E+04	±2.18E+04
Lactobacillus spp. H_2O_2-									
Vagina	1.60E+06	4.34E+04	6.37E+05	7.00E+04	8.34E+04	7.85E+04	2.96E+04	1.73E+05	1.25E+05
	±4.96E+06	±3.81E+04	±3.07E+05	±1.75E+04	±2.51E+04	±2.26E+04	±7.75E+04	±3.95E+05	±3.33E+05
Ainus	7.57E+04	1.66E+05	7.13E+04	1.86E+04	2.56E+06	1.62E+06	2.86E+05	5.37E+04	1.34E+05
	±2.74E+04	±1.89E+05	±2.23E+04	±4.75E+04	±1.20E+06	±9.60E+06	±6.33E+05	±9.56E+04	±3.95E+05
Bifidobacterium spp.									
Vagina	1.33E+03	4.39E+00	5.02E+02	1.00E+04	0.00E+00	3.68E+03	0.00E+00	0.00E+00	0.00E+00
	±4.99E+03	±1.89E+02	±3.05E+02	±3.61E+04	±0.00E+00	±2.24E+04	±0.00E+00	±0.00E+00	±0.00E+00
Ainus	6.55E+04	1.18E+05	8.61E+04	1.92E+05	8.06E+04	1.21E+05	1.41E+05	7.97E+04	1.00E+05
	±1.15E+04	±3.00E+05	±1.51E+05	±5.09E+05	±1.92E+04	±3.49E+05	±1.82E+05	±2.24E+04	±1.13E+05
Candida spp.									
Vagina	1.79E+04	1.43E+03	7.67E+03	3.36E+02	3.34E+03	2.24E+03	2.40E+02	4.32E+04	2.89E+04
	±6.47E+04	±5.44E+03	±3.97E+04	±8.33E+02	±8.38E+03	±6.83E+03	±5.99E+02	±1.78E+04	±1.47E+04
Anus	1.13E+03	4.86E+01	5.22E+02	4.29E+00	8.39E+02	5.32E+02	6.00E+00	1.00E+00	2.67E+00
	±3.98E+03	±6.22E+01	±5.47E+02	±1.55E+01	±4.00E+03	±3.20E+03	±1.28E+01	±4.36E+00	±8.54E+00

We showed that there were no significant differences in the numbers of the hydrogen-peroxide positive/negative *Lactobacillus* spp., *Bifidobacterium* spp. and *Candida* spp. in the vagina and rectum in patients colonized with group B streptococci (n=15) and uncolonized women (n=27). The results are shown in Table 2.

DISCUSSION

Despite the introduction of appropriate prophylaxis in the US and many European countries, group B Streptococcus is still a major cause of infant morbidity and mortality in developed countries. Acquired experiences indicate that the most effective method of limiting the number of infections in newborns is the introduction of screening for all pregnant women for GBS carriage and, should a positive result be obtained, implementing targeted perinatal antibiotic prophylaxis. It is a well-known fact that the most significant factor predisposing newborns to the development of infection is the presence of *S. agalactiae* in the mother's genital or gastrointestinal tract, from where the bacteria are transmitted to the baby.

Therefore, it is advised to carry out microbiological examination of the vagina and anus in women between the 35th and 37th weeks of pregnancy. As GBS colonization is often transitory, predicting on the grounds of a culture performed earlier than 5 weeks before delivery is fallible and recommended only in special cases, i.e. in women with a threat of premature labour or with premature rupture of membranes (Melin *et al.,* 2011; Schrag *et al.,* 2002; Verani *et al.,* 2010).

Most studies of vaginal microbial flora employed a cross-sectional study design in which individuals are sampled at one discrete time point. As a result, little is known about the temporal dynamics of vaginal bacterial communities, for example during pregnancy. In normal pregnancy, the resident vaginal flora is thought to provide protection against infection by a number of complementary mechanisms (Klebanoff *et al.,* 1991; van Belkum *et al.,* 2007). Much of our knowledge of the composition of the female genitourinary tract and rectum comes from qualitative and semiquantitative descriptive studies using cultivation-dependent techniques.

In our study, 42 healthy pregnant women were monitored in three subsequent trimesters of pregnancy for quantitative determination of *S. agalactiae* (GBS) and other components of the normal flora colonizing the vagina and anus. Analysis of the results confirmed that vaginal and rectal ecosystems observed during testing are dynamic and that they change over time. We demonstrated a wide variability in the GBS population in women from the study group. The observed GBS colonization was continuous, intermittent, or periodic in nature, depending on the examined patient. The vaginal colonization changed from 24% in the first trimester to 17% in the third, while the anal colonization fluctuated from 19% in the first to 24% in the second and 17% in the third trimester. The extent of GBS colonization, determining the number of the colony forming units per 1 ml, demonstrated large fluctuations in relation to the study of anatomical side and trimester between individuals.

According to CDC, a routine screening for asymptomatic bacteriuria is recommended in pregnant women, and laboratories should screen urine culture specimens for the presence of GBS in concentrations of 10^4 cfu/ml or greater. *S. agalactiae* is found in the urine of 2% to 7% of pregnant women (Verani *et al.,* 2010). We demonstrated that 5% of the pregnant women had asymptomatic GBS bacteriuria which constituted 13% of the GBS-positive women. These relatively high results may be connected with the high percentage of GBS colonization among the women surveyed. Moreover, the noted cases of bacteriuria were strongly connected to a massive colonization by GBS in the vagina and anus during the subsequent trimesters of pregnancy. This confirms that GBS bacteriuria in a pregnant woman is a marker for heavy genital tract colonization (Verani *et al.,* 2010).

Using PFGE method and serotyping, we analysed five different colonies of GBS isolated from all positive materials. We demonstrated that more than two thirds of the GBS-positive women studied were colonized during their pregnancy by a single GBS clone which belonged to one of the serotypes. Similar results were previously described by Perez-Ruiz *et al.* (2004) who analysed 15 colonies of *S. agalac-*

tiae isolated from each of the 30 vaginal-rectal colonized women. Our results confirm that in most cases of periodic vaginal–rectal colonization of pregnant women by GBS the genetically identical GBS strain was determined in the next study period. Using molecular typing methods, we demonstrated the same GBS clones occurring periodically or permanently in the genitourinary tract and anus of all GBS-positive women. In a few cases we showed that the same GBS strains were present in the puerperium despite the antibiotic prophylaxis applied during labour. Only one patient showed a different PFGE type of GBS colonization in the puerperium than the one that was marked during pregnancy. This proves that the reservoir of the strains was determined in the gastrointestinal tract of the patients tested.

The real-time PCR (qPCR) method based on amplification of a *cfb* gene fragment of GBS was described by Ke *et al.,* (2000). Quantitative PCR may offer the advantage of reduced time-to-results, making it useful as an intrapartum screening method (El Aila *et al.,* 2011; Verani *et al.,* 2010). Using the qPCR method, we obtained values higher by one or two orders of magnitude compared to culture method results, and we also obtained positive results for 7% of materials negative in the culture. The number of GBS-positive established by qPCR in relation to the cultured method is in agreement with that reported in the literature (El Aila *et al.,* 2011). Yet, in the case of urine samples, results from the qPCR method were lower. This was probably associated with polymerase inhibitors which are present in urine samples and affect the amplification and lower the sensitivity of the method.

Lactobacilli are the predominant vaginal microorganisms in healthy adult women and form a critically important component of the vaginal ecosystem. Lactobacilli, mainly the strains that are hydrogen peroxide (H_2O_2)-producing, may have a protective effect against colonization of a woman's vagina by different pathogens. Few studies have identified lactobacilli to species level and detected H_2O_2 production (Strus et *et al.,* 2006; Wilks *et al.,* 2004). H_2O_2-positive lactobacilli have been demonstrated to play a protective role in bacterial vaginosis (BV) and urinary tract infections (UTI) (Klebanoff *et al.,* 1991; Strus *et al.,* 2006). However, little is known on regulation of the GBS population by lactobacilli *in vivo*. Our previous *in vitro* pilot study showed a great ability of H_2O_2-negative *L. plantarum* C 11 strain which produces plantaricin to inhibit the GBS growth (Bodaszewska-Lubas *et al.,* 2012). We demonstrated that all women studied in the third trimester of pregnancy had H_2O_2-positive lactobacilli in their vaginas; moreover, 27% of pregnant women had H_2O_2-negative lactobacilli in vaginal smears. The study by Petricevic *et al.* (2012) supports the hypothesis that the rectum may play an important role as a reservoir for some strains of lactobacilli that colonise the vagina. Our findings seem to confirm this hypothesis.

Maternal colonization by *Bifidobacterium* spp. in the gastrointestinal tract had the most consistent effects on the infant's bifidobacterial microbiota (Gronlund *et al.,* 1999). To our knowledge, there are no studies reporting the changes of the vagina and anus in *Bifidobacterium* genus in pregnant women. In our study, *Bifidobacterium* spp. occurred in anal smears in half of the studied women, but were almost absent in the vagina.

Up to 40% of pregnant women may demonstrate vaginal colonization by *Candida* spp., a twofold increase from the prevalence rate in non-pregnant patients. This is believed to be powered by increased levels of circulating oestrogens and deposition of glycogen and other substrates in the vagina during pregnancy (Hay *et al.,* 2007). In our study, the colonization rate of *Candida* spp. in vagina during pregnancy slightly increased from 27% in the first trimester to 30% in the third, wherein the women included in the study did not show symptoms of vulvovaginitis. These results are very similar to Leli *et al.* 's research (2013). They indicated that colonization by *Candida* species in pregnant women equalled 31% and was significantly more often asymptomatic as compared to non-pregnant patients (Leli *et al.,* 2013).

El Aila *et al.* (2009b) identified and genotyped bacteria from paired vaginal and rectal samples from pregnant women. They confirmed that there was a certain degree of correspondence between the vaginal and rectal microflora, not only with regard to species composition but also with regard to the strain identity (El Aila

et al., 2009b). Our study yielded similar results connected to *S. agalactaie* strains and other normal flora components, such as *Lactobacillus* spp., *Bifidobacterium* spp. and *Candida* spp., isolated from vaginal and rectal smears.

We demonstrated that there were no significant differences in the frequency of isolation of any species or bacterial counts between the vaginal and rectal smears in pregnant women colonized and uncolonized by GBS. Similar results were reported by Gustafsson *et al*. (2011) who investigated the Lactobacillus flora in vagina and rectum of fertile and postmenopausal healthy Swedish women. There was no statistical significant fluctuation of the number of bacteria in the rectal and vaginal smears between fertile and postmenopausal women. But similarly to that study we observde a certain degree of correspondence between the vaginal and rectal microflora (Gustafsson *et al.,* 2011).

In summary, the analysis of women in three consecutive trimesters of pregnancy on the basis of a study group and control group showed no statistically significant differences in either the species (qualitative) or quantitative composition in vaginal and rectal flora in both of the groups. Therefore, GBS should be considered as a component of the microbiota and an opportunistic microorganism rather than a typical pathogen, because it does not distort the composition of the normal flora of the genital tract of women.

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