ORIGINAL ARTICLE

Presence and resistance of *Streptococcus agalactiae* in vaginal specimens of pregnant and adult non-pregnant women and association with other aerobic bacteria

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ABSTRACT

Aim To determine the prevalence rate and resistance profile of *Streptococcus agalactiae* (*S. agalactiae)* in vaginal swabs of pregnant and adult non-pregnant women in the Tuzla region, Bosnia and Herzegovina (B&H), as well as its association with other aerobic bacteria.

Methods This prospective study included 200 women, 100 pregnant and 100 adult non-pregnant. The research was conducted at the Institute of Microbiology, University Clinical Center Tuzla from October to December 2015. Standard aerobic microbiological techniques were used for isolation and identification of *S. agalactiae* and other aerobic bacteria. Antimicrobial susceptibility was determined by the disk diffusion and microdilution method (VITEK 2/AES instrument).

Results Among 200 vaginal swabs, 17 (8.50%) were positive for *S. agalactiae*, e. g., 7% (7/100) of pregnant and 10% (10/100) of adult non-pregnant women. In the pregnant group, 71.4% (5/7) of *S. agalactiae* isolates were susceptible to clindamycin and 85.7% (6/7) to erythromycin. In the adult non-pregnant group, only resistance to clindamycin was observed in one patient (1/10; 10%). *S. agalactiae* as single pathogen was isolated in 57.14% (4/7) of pregnant and 60% (6/10) of adult non-pregnant *S. agalactiae* positive women. In mixed microbial cultures *S. agalactiae* was most frequently associated with *Enterococcus faecalis* and *Escherichia coli.*

Conclusion The rate of *S. agalactiae* positive women in the population of pregnant and adult non-pregnant women of Tuzla Canton, B&H is comparable with other European countries. Large studies are needed to develop a common national strategy for the prevention of *S. agalactiae* infection in B&H, especially during pregnancy.

Key words: vaginal diseases, reproductive tract infections, bacterial infections

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Original submission:
08 September 2016;
Revised submission:
03 October 2016;
Accepted:
05 October 2016
doi: 10.17392/876-16

Med Glas (Zenica) 2017; 14(1):98-105

INTRODUCTION

Streptococcus agalactiae (*S. agalactiae*) (group B streptococcus, GBS) is a Gram–positive bacterium that often asymptomatically colonizes healthy adults. *S. agalactiae* is present as a part of the normal microflora of the female genital tract (25% of healthy women) and the lower gastrointestinal tract (1, 2). However, this opportunistic bacterium can effectively adapt to changes in the host environment, express virulence factors which enable it to break the innate immunity barrier of the host, and cause serious invasive diseases and tissue damage (2-4). A very important virulence factor of *S. agalactiae* clinical isolates is a capsular polysaccharide antigen rich in sialic acid, although in the pathogenesis of infection other antigens may play important roles as well (5). Based on the capsular antigen, *S. agalactiae* is divided into ten serotypes labelled as Ia, Ib, II – IX (4).

S. agalactiae is an important infectious agent in pregnant women and adult non pregnant women (age 20-59). Genital tract colonization with *S. agalactiae* of adult women varies between 5-40% depending on the age, health and physiological status of tested female population (6,7). The incidence of invasive diseases caused by this bacterium is increasing in women of reproductive age who are not pregnant (age 15-44) and with some co-morbidities such as diabetes mellitus. In the study of Edwards and Baker 88% of female adults with *S. agalactiae* infection had at least one underlying illness, with diabetes mellitus present in 44% of cases (2).

In pregnant women, the rate of GBS colonization is around 5-30%, and the presence of these bacteria during pregnancy is often clinically asymptomatic (7-10). Vaginal environment provides optimal conditions for the growth and proliferation of *S. agalactiae*, which can have detrimental consequences for both a mother and her baby (9). Infection in late pregnancy is one of the risk factors for poor pregnancy outcome since *S. agalactiae* has the ability to penetrate intact amniotic membranes causing amnionitis, affecting fetus *in utero*. As an invasive pathogen, *S. agalactiae* infiltrates different tissues and body cavities such as intrauterine space, lungs of neonates and other organs including brain (1). *S. agalactiae* infection during pregnancy can lead to stillborn birth or abortion (2,8).

Globally, *S. agalactiae* is recognized as one of the leading causes of neonatal sepsis, pneumonia and meningitis, thus represents an important cause of neonatal morbidity and mortality (4,6). *S. agalactiae* transmission from a colonized mother to her newborn can occur *in utero* by the ascending infection or during birth when an infant aspirates contaminated amniotic and/or vaginal fluid (2). Interestingly, even though *S. agalactiae* is the leading cause of perinatal infections and postpartum septicemia, incidence of the disease is extremely disproportionate to the incidence of colonization, which depends on the method of cultivation of bacteria and collection of samples. It has been suggested that the greater number of *S. agalactiae* present, meaning the greater colonization density, the greater the likelihood of a disease (11).

The Centers for Disease Control and Prevention (CDC) in its guidelines for the prevention of neonatal infections suggests intrapartum chemoprophylaxis during delivery to women at risk for transmitting *S. agalactiae* to their newborns (12,13). Systemic administration of antimicrobial drugs before the start of labor or membrane rupture is very effective in reducing neonatal colonization, lowering early neonatal *S. agalactiae* infections to less than 50% of levels before prophylaxis (14). In some countries of the world that implement active prevention measures the incidence of these neonatal infections has significantly decreased. According to the CDC 2000 report, the overall incidence of early onset of the disease from 1998 to 2000 was 0.5-0.6 cases per 1000 live births, with geographical and racial variations (15). *S. agalactiae* is still sensitive to penicillin and other β-lactam antibiotics, and the resistance to antibiotics such as macrolides, lincosamide and quinolones, which are used as an alternative therapy has been recorded worldwide (16). However, there have been reports of enhanced minimum inhibitory concentrations (MICs), near the upper limit of sensitivity, to penicillin or ampicillin of some invasive isolates (10,13). This decreased sensitivity to penicillins should not be necessarily considered as resistance, although it is somewhat worrisome for it indicates a potential development of true resistance of GBS isolates to penicillins, and it requires monitoring (6).

Despite these clear prevention guidelines, in the Western world *S. agalactiae* remains the major

cause of infectious etiology of neonatal morbidity and mortality (7). There are no published data on the rate of *S. agalactiae* infection in women in Bosnia and Herzegovina (B&H), either for pregnant or adult non-pregnant women. Moreover, despite the established link between *S. agalactiae* infection and its effect on a mother and her baby *in utero* and postpartum, the CDC recommendations on control and prevention of *S. agalactiae* infections are generally not implemented in Bosnia and Herzegovina.

The aim of this study was to determine the prevalence rate of *S. agalactiae* in vaginal swabs of pregnant and adult non-pregnant women in the Tuzla region, Bosnia and Herzegovina, as well as their antimicrobial susceptibility pattern and its association with other aerobic bacteria.

MATERIALS AND METHODS

Patients and study design

This prospective study was conducted at the Institute of Microbiology, University Clinical Center (UCC) Tuzla in the period from October to December 2015. Specimens received for microbiological analysis were collected at one of the health institutions in the municipalities of Tuzla Canton (Banovići, Čelić, Kladanj, Gračanica, Teočak, Sapna) and at the Clinic for Gynecology and Obstetrics, UCC Tuzla. A lower vaginal swab was taken from each patient. Basic demographic data of each respondent (name, year of birth, place of residence, leading diagnosis) were taken from referrals received from gynecologists of the aforementioned health institutions.

The study included consecutive 200 female inpatients and outpatients diagnosed with colpitis. Based on a physiological state and a referral diagnosis, patients were divided into two groups. The first group was composed of 100 pregnant women (ages 15-44), and the second group of 100 adult women (ages 20-59) who were not pregnant. The women were grouped according to the WHO classification, e. g., women of 15-44 years represented females of child bearing age, termed "pregnant women" and women of 20-59 years old were termed "adult women" ("adult non-pregnant" women in this study) (17).

Microbiological analysis

Each sample was plated and cultured on blood (Liofilchem, Italy) and chocolate agar (Liofilchem, Italy), chromogenic medium CPS (bioMérieux, Marcy l'Étoile, France) and glucose broth (Liofilchem, Italy). All three plates were incubated overnight at 37 °C under aerobic conditions. Subcultivation of glucose broth on blood agar plates was carried out after overnight incubation at 37 °C, only if the primary plates remained sterile. From each β-hemolytic, catalase-negative colony, a microscopic slide was prepared and stained with Gram stain. Further identification was performed only for samples containing gram-positive cocci.

In order to isolate a suspected *S. agalactiae*, CAMP test was performed (with β-hemolytic *Staphylococcus aureus* ATCC 25923 as a reference strain). After a positive CAMP test, identification was confirmed by an automated instrument for ID/AST testing, VITEK 2 Compact AST-GP ID card (bioMérieux, Marcy l'Étoile, France), according to the manufacturer's instructions.

Identification of other bacterial species and fungi presented in swab specimens was carried out according to standard microbiological procedures (18).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of *S. agalactiae* isolates was performed by disk diffusion method as recommended by CLSI (19), and microdilution method on VITEK 2/AES instrument (AST-586 card).

Eight antibiotic disks (Liofilchem, Italy) were used: ampicillin (10 μg), clindamycin (2 μg), erythromycin (15 μg), cefepime (30 μg), cefotaxime (30 μg), vancomycin (30 μg), levofloxacin (5 μg), linezolid (10 μg) (19).

Statistical analysis

A statistical analysis was performed using $\chi 2$-test and Student t-test. Statistical level of 95% ($p<0.05$) was considered as significant.

RESULTS

From October to December 2015, lower vaginal swabs of 200 female patients (100 pregnant women at different stages of pregnancy and 100 adult non-pregnant women) received at the Institute of Microbiology, UCC Tuzla, were examined.

The mean age of pregnant women was 27.74 years, ranging 17-44 years. Most pregnant women were 29 years old or younger (66/100, 66%).

32/100 (32%) were 25-29 years old, 34/100 (34%) 15-24 years, 18/100 (18%) 30-34 years, and 8/100 (8%) 35-39 and 40-44 years old each.

Adult non-pregnant women were evenly distributed across different age groups, with an average age of 37.03, ranging from 20-59 years. Age distribution of adult non-pregnant women was: 10/100 (10%) were 20-24 years old, 21/100 (21%) were 25-29, 13/100 (13%) were 30-34, 17/100 (17%) were 35-39, 14/100 (14%) were 40-44, 10/100 (10%) were 45-49 and 15/100 (15%) were ≥ 50 years old.

S. agalactiae was detected in lower vaginal swabs of 7% (7/100) pregnant women and 10% (10/100) adult non-pregnant women. Twenty-one percent (21/100) of swab specimens from pregnant and 22% (22/100) from adult non-pregnant women were sterile. Non-pathogenic bacteria were isolated from 25% (25/100) and 17% (17/100) of pregnant and adult non-pregnant women, respectively. Besides *S. agalactiae*, other microbial pathogens were detected in 47% (47/100) isolates of pregnant and 51% (51/100) of adult non-pregnant women (Table 1).

Table 1. Distribution of bacterial isolates of lower vaginal swabs of pregnant and adult non-pregnant women

Microorganism(s)	No (%) of women		
	Pregnant (n=100)	Adult non-pregnant (n=100)	Total
S. agalactiae	4	6	10
S. agalactiae + *C. albicans*	1	1	2
S. agalactiae + *E. faecalis*	-	2	2
S. agalactiae + *E. coli*	1	1	2
S. agalactiae + *E. coli* + *C. albicans*	1	-	1
Other*	47	51	98
Non-pathogenic†	25	17	42
Sterile	21	22	43
Total	100	100	200

**E. faecalis*, *E. coli*, *E. faecium*, *Enterobacter* spp., *Pseudomonas* spp., *C. albicans*, *K. pneumoniae*, *K. oxytoca*, *M. morganii*, *S. aureus*
†coagulase-negative staphylococci, diphtheroids

In 57.14% (4/7) of *S. agalactiae*-positive pregnant women *S. agalactiae* was detected as a single pathogen and in 42.86% (3/7) *S. agalactiae* was isolated in combination with *Candida albicans* (*C. albicans*) and/or *Escherichia coli* (*E. coli*). In adult non-pregnant women, *S. agalactiae* was present as a single pathogen in 60% (6/10) and in 40% (4/10) women along with either *Enterococcus faecalis (E. faecalis*), *E.coli* or *C. albicans*. There was no statistically significant difference between the prevalence of *Streptococcus agalactiae* isolates from pregnant and adult non-pregnant women (p=0.6133) (Table 1).

Prevalence of *S. agalactiae* in lower vaginal swabs of women whose samples were positive for any pathogenic microorganism, was 12.96% (7/54) for pregnant and 16.39% (10/61) for adult non-pregnant women.

The average age of women positive for *S. agalactiae* in the pregnant group was 24.85 (ranging 20-29 years) and in adult non-pregnant women it was 40.2 (ranging 27-53 years). All but one women (age 27) in the adult non-pregnant *S. agalactiae*-positive women were aged 38 and older (p<0.05).

Among other aerobic pathogens presented in lower vaginal swab specimens, in both pregnant and adult non-pregnant women *E. faecalis* as single pathogen was most frequently presented (25% and 26%, respectively) (Table 3). In both groups pure culture of *E. coli* was detected in only a small number of samples (1% and 3%, respectively), while *C. albicans* was presented in 9% and 10%, respectively. One isolate of pregnant women had *Klebsiella pneumoniae* (*K. pneumoniae*) as a single pathogen, while in adult non-pregnant women *Enterococcus faecium* (*E. faecium*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Enterobacter* spp. were detected as a single pathogen in one isolate each (1%). A combination of two or three pathogenic microorganisms was detected in 9% (9/100) and 10% (10/100) of pregnant and adult non-pregnant women (Table 2).

Table 2. Distribution of microorganisms other than *S. agalactiae* presented in vaginal samples of pregnant and adult non-pregnant women

Microorganism(s)	No (%) of women		
	Pregnant (47)	Adult non-pregnant (51)	Total
Enterococcus faecalis	25 (53.19)	26 (50.98)	51 (52.04)
Enterococcus faecium	-	1 (1.96)	1 (1.02)
Escherichia coli	1 (2.12)	3 (5.88)	4 (4.08)
Klebsiella pneumoniae	2 (4.25)	-	2 (2.04)
Candida albicans	10 (21.27)	9 (17.64)	19 (19.38)
Enterobacter spp.	-	1 (1.96)	1 (1.02)
Pseudomonas spp.	-	1 (1.96)	1 (1.02)
E. faecalis + *E. coli*	2 (4.25)	6 (11.76)	8 (8.16)
E. faecalis + *K. pneumoniae*	1 (2.12)	1 (1.96)	2 (2.04)
E. faecalis + *Klebsiella oxytoca*	1 (2.12)	-	1 (1.02)
E. faecalis + *Staphylococcus aureus*	1 (2.12)	-	1 (1.02)
E. faecalis + *Morganella morganii*	1 (2.12)	-	1 (1.02)
E. faecalis + *C. albicans*	3 (6.38)	2 (3.92)	5 (5.10)
E. coli + *K. pneumoniae*	-	1 (1.96)	1 (1.02)
Total	47 (100)	51 (100)	98 (100)

In the pregnant group, 71.4% (5/7) of isolates were susceptible to clindamycin and 85.7% (6/7) to erythromycin. One of the isolates was resistant to clindamycin and erythromycin. In the group of adult non-pregnant women, only resistance to clindamycin was observed in one isolate (1/10; 10%) (Table 3).

Table 3. Antimicrobial susceptibility of *Streptococcus agalactiae* isolates obtained from pregnant and adult non-pregnant women

	Percentage (%) of susceptibility	
Antibiotic	**Pregnant**	**Adult non-pregnant**
Ampicillin	100.0	100.0
Clindamycin	71.4	90.0
Erythromycin	85.7*	100.0
Cefepime	100.0	100.0
Cefotaxime	100.0	100.0
Vancomycin	100.0	100.0
Levofloxacin	100.0	100.0
Linezolid	100.0	100.0

*in the pregnant group, the same *S. agalactiae* resistant to clindamycin was also resistant to erythromycin

DISCUSSION

Vagina and its unique microflora form a delicately balanced ecosystem, where the environment inside vagina controls the type of microbes present, and in return this microflora controls the vaginal environment (20). This ecosystem is dynamic, constantly changing dependent on the anatomy and composition, which is directed by woman's age, menopause, the day of a menstrual cycle, pregnancy, infections, use of birth controls, frequency of sexual relations, number of partners, as well as habits and practices, such as irrigation (21).

It is generally accepted that *Lactobacilli* are the predominant members of post-adolescence vaginal flora (22). Variety of other microorganisms such as *Staphylococcus, Ureaplasma, Corynebacterium, Streptococcus, Peptostreptococcus, Gardnerella, Bacteroides, Mycoplasma, Enterococcus, Escherichia, Veillonella, Bifidobacterium* and *Candida* may be present but in significantly lower numbers. The identity and diversity of this population is to a great extent masked by the complexed interaction of different players of vaginal flora, which are still not clearly defined (23). Qualitative studies of bacteriological assessment of vaginal swabs obtained from asymptomatic women showed that *S. agalactiae* is often isolated with lactobacilli, *Gardnerella vaginalis*, coagulase-negative staphylococci and enterococci. Yet, not all the studies agree with this finding (24). Thus, Carson et al. have found that when *S. agalactiae* is isolated no other group of *Streptococcus* species is present. Also, when any β-hemolytic *Streptococcus* species is present, *S. agalactiae* is not present as an associated flora (23). In the present study it was found that in both groups of women the greatest number of *S. agalactiae* isolates were presented in pure cultures, while two *S. agalactiae* positive samples from the adult non-pregnant group were isolated along with *E. faecalis*, and in each group one culture of *S. agalactiae* also contained *E. coli* and *C. albicans*. In previous work on the prevalence of bacterial vaginosis among sexually active women in Tuzla Canton, Numanović et al. found that besides *Gardnerella vaginalis*, most frequently isolated bacteria from vaginal swabs were *Enterococcus foecalis* (26.95%) and *Escherichia coli* (23.91%), while *S. agalactiae* was isolated from 10% of samples (25). Presence of *S. agalactiae* in the same culture with other bacteria, such as *E. coli* and *E. faecalis* could be an indication of a possible aerobic vaginitis (24).

Data on the presence of *S. agalactiae* in vaginal specimens from different countries vary depending on the characteristics of the studied population of women. In a retrospective study conducted in Osijek (Croatia) from 118 isolates of pregnant women *S. agalactiae* was found in 24.6% (7). Very similar data (25.3%) were obtained by Shabayek et al. in Egypt (26). A lower incidence in pregnant women of 10.6% was found in Manisi (Turkey), with a significantly lower rate of *S. agalactiae* positive samples among patients of 24 years or older (27). In our study, microbiological examination of vaginal swab specimens of pregnant and adult non-pregnant women diagnosed with colpitis showed *S. agalactiae* in 7% and 10% of samples, respectively (8.5%, 17/200 in total sample).

Furthermore, prevalence of *S. agalactiae* in the vaginal swabs depends on the method of sample collection, as well as the method of isolation and identification (28). If sample collection is carried out outside a microbiological laboratory it is recommended to use an adequate transport media. In our research, 21% and 22% of sterile samples of pregnant and adult non-pregnant women, respectively, were found, which is an indication of improper sampling/transport. It is recommended that if the isolation of *S. agalactiae* is made from primary sterile materials, a non-selective medium, such as blood agar, should be used. However, the

standard for the isolation of *S. agalactiae* included a selective liquid medium, such as Todd-Hewitt or Lim broth, containing antibiotics such as nalidixic acid and colistin or gentamicin, used to inhibit the growth of other bacteria present (29). Besides standard tests, such as Gram stain, catalase and CAMP test, definitive identification of *S. agalactiae* is based on a serological test, which proves the presence of polysaccharide group-specific antigen present on all types of *S. agalactiae* strains (29). The main limitation of *S. agalactiae* detection by culture is the length of cultivation and identification (29). In our report, microbiological assessment of vaginal swabs was performed using blood and chocolate agar, chromogenic medium CPS and glucose broth because none of the samples that were received in our laboratory had a referral for *S. agalactiae* screening. The identification process we conducted was identical to the recommendations of the aforementioned authors (28).

A significant rate of resistance among *S. agalactiae* isolates, which has been increasing over the past 20 years has been reported for some antibiotics that have been used as an alternative therapy for treatment of *S. agalactiae* infections, such as macrolides, lincosamides and fluoroquinolones (16). The alternative antibiotic choices, usually for women with reported penicillin allergies, have traditionally been erythromycin or clindamycin. Resistance to erythromycin has frequently been associated, but not always, with cross-resistance to clindamycin (10,16). Examining the susceptibility of 4800 isolates of *S. agalactiae* it has been found that 32% of them were resistant to erythromycin, 15% to clindamycin, while 99% of clindamycin resistant isolates were also resistant to erythromycin (30). Very similar data have been published by other authors, showing no resistance to penicillin and ampicillin among examined isolates of *S. agalactiae*, but reported resistance to erythromycin 13.5% - 21.2% and 9.1% - 23.68% to clindamycin (26, 27). *S. agalactiae* isolates examined in our study were susceptible to most antibiotics tested. Two exceptions were isolates from pregnant group (one isolate was resistant to clindamycin, other was non-susceptible to either clindamycin or erythromycin). Also, one isolate from the adult non-pregnant women group exhibited resistance to clindamycin.

In conclusion, our study has shown a similar rate of *S. agalactiae* infection among pregnant and adult non-pregnant women comparable to the results from other clinical centers. None of the samples that were received in our laboratory had a referral for *S. agalactiae* screening, thus the cultivation methods used were not specifically directed towards *S. agalactiae* detection. Besides a very low number of isolates analyzed in this study, the main limitation is the inability to use a selective medium. In light of the possibility that the percentage of positive samples could have been higher if a different cultivation method was used, it is advisable to start performing regular screening for *S. agalactiae* especially for the population of fertile and pregnant women. Larger studies are needed for the development and wide implementation of a national strategy for the prevention of *S. agalactiae* infection in Bosnia and Herzegovina, as well as greater compliance with the CDC recommendations.

ACKNOWLEDGEMENTS

We thank the staff at the Institute of Microbiology, UCC Tuzla for their assistance.

FUNDING

No specific funding was received for this study.

TRANSPARENCY DECLARATION

Competing interests: none to declare.

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Zastupljenost i rezistencija *Streptococcus agalactiae,* izolovanih iz vaginalnih uzoraka kod trudnica i žena odrasle dobi koje nisu trudne, te njegova udruženost s drugim aerobnim bakterijama

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SAŽETAK

Cilj Odrediti stopu zastupljenosti i rezistenciju *Streptococcus agalactiae* (*S. agalactiae*), izolovanih iz vaginalnog brisa kod trudnica i žena odrasle dobi u Tuzlanskom kantonu, Bosna i Hercegovina (BiH), kao i njegovu udruženost s drugim aerobnim bakterijama.

Metode Prospektivno istraživanje obuhvatilo je 200 žena (100 trudnica i 100 odraslih žena koje nisu bile trudne). Istraživanje je provedeno u Zavodu za mikrobiologiju Univerzitetskog kliničkog centra Tuzla, od oktobra do decembra 2015. godine. Za izolaciju i identifikaciju *S. agalactiae* i drugih aerobnih bakterije korištene su standardne mikrobiološke metode. Antimikrobna osjetljivost ispitana je disk-difuzionom i mikrodilucionom metodom (VITEK 2/AES aparat).

Rezultati Od 200 pregledanih vaginalnih briseva, 17 (8,50 %) uzoraka bilo je pozitivno na *S. agalactiae*, 7% (7/100) kod trudnica i 10% (10/100) kod žena odrasle dobi koje nisu bile trudne. Osjetljivost na klindamicin zabilježena je u 71,4% (5/7) izolata kod trudnica, a u 85,7% (6/7) na eritromicin. U grupi žena odrasle dobi koje nisu trudne, 10% (1/10) izolata bilo je rezistentno na klindamicin. Kod trudnica je u 57,14% (4/7) slučajeva, a kod žena odrasle dobi u 60% (6/10) slučajeva, *S. agalactiae* izolovan u čistoj kulturi, a od ostalih aerobnih bakterija najviše su bili udruženi s *Enterococcus faecalis* i *Escherichia coli*.

Zaključak Stopa *S. agalactiae*-pozitivnih žena u populaciji trudnica i žena odrasle dobi koje nisu trudne u Tuzlanskom kantonu, BiH, uporediva je s drugim evropskim zemljama. Neophodno je razviti zajedničku nacionalnu strategiju u BiH u prevenciji infekcija genitalnog trakta *S. agalactiae,* a posebno u toku trudnoće.

Ključne riječi: vaginalne bolesti, infekcije reproduktivnog trakta, bakterijske infekcije