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VAGINAL MICROFLORA DURING PREGNANCY AND ITS TRANSMISSION TO NEWBORN

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TARTU UNIVERSITY
PRESS

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by Roman numerals (I–VI), and on some unpublished data:

I Mändar R and Mikelsaar M. (1996). Vaginal microflora during pregnancy. *Alpe Adria Microbiology Journal* 1: 41–50. This Journal is indexed by Excerpta Medica EMBASE.

II Mändar R, Saag H, Peil P and Mikelsaar M. (1995). Bacterial vaginosis during pregnancy. *Microecology and Therapy* 23: 24–31.

III Mändar R. and Mikelsaar M. (1996). Transmission of mother's microflora to the newborn at birth. *Biology of the Neonate* 69: 30–35.

IV Mändar R. (1993). Bacterial vaginosis. *Estonian Physician* 1: 15–17 (in Estonian).

V Mikelsaar M and Mändar R. (1993). Development of individual lactic acid microflora in the human microbial ecosystem. In: *Lactic Acid Bacteria* (Eds S Salminenen, A von Wright). Marcel Dekker Inc., New York, pp 237–238, 244–248, 256–259.

VI Mändar R, Türi M, Allen SD, Mikelsaar M. Susceptibility patterns of vaginal lactobacilli isolated from pregnant women in Estonia and USA. In Manuscript.

ABBREVIATIONS

BV	bacterial vaginosis
TAb	threatened abortion
VMf	vaginal microflora
CFU/swab	colony forming units per swab
STD	sexually transmitted diseases
PROM	premature rupture of membranes
AMT	antimicrobial treatment
HT	hormonal treatment
APO	adverse pregnancy outcome
GBS	Group B streptococci *seu Streptococcus agalactiae*
G–	Gram-negative
G+	Gram-positive
G-var	Gram-variable

1. INTRODUCTION

There exist predictable relationships between specific microbial species and higher life-forms, in which both sides are interactive. An example of these relationships is normal human microflora. The unsolved questions — how it changes over time, the factors governing it, and the biological effects of its disruption — make the study of microbial ecology important for the host's health and disease. However, it is quite difficult to evaluate the state of the normal microflora because of its extreme complexity and individuality. The need for finding new evaluation principles has been repeatedly underlined at several meetings of the Society for Microbial Ecology and Disease (Gorbach, 1992; Ahtonen *et al.*, 1993; Onderdonk, 1994; Mehta *et al.*, 1995).

It has been postulated that women are dependent on the normal vaginal microflora ecosystem for maintaining a healthy female organism. When the ecosystem is disrupted by either altering the host tissues or the composition of the flora, some diseases may result (Larsen 1993; Overman, 1993; Krohn *et al.*, 1995; Lee *et al.*, 1995). Since the VMf is a complex ecosystem, it has to be described in multiple aspects — interactions between different microorganisms, between the microorganisms and macroorganism, between microorganisms and environmental factors, as well as dynamic changes in the course of time, *etc.* As regards gastrointestinal microecology, a new approach for evaluating the system was recently proposed (Mikelsaar, 1992). An auxiliary computer program for clinical application of this method has been developed (Mändar *et al.*, 1992; Mikelsaar *et al.*, 1995). Similar approach to vaginal microflora (VMf) would be necessary for finding out what changes may occur there under different physiological conditions and when the large variety of normal state might change into pathological abnormality. At University of Tartu many studies on the vaginal lactoflora have been performed (Lenzner, 1964; 1973; Lenzner *et al.*, 1984; Brilene *et al.*, 1989; Brilene, 1990). However, there is a lack of knowledge about the interactions between different vaginal microorganisms, *e.g.* vaginal microbial ecosystem of Estonian fertile women over long periods. What is more, some substantial differences in gastrointestinal microfloras have been described in reference to different geographical areas, unassociated with the ethnic groups or climate (Salminen *et al.*, 1995), hence, the same could be expected also in case of vaginal microflora.

It is especially important to check on the state of the VMf during pregnancy, as it may affect both the woman's and her newborn's health. The mother's delivery tract's microflora forms the basis for the development of the baby's indigenous microflora (Mikelsaar *et al.*, 1989; Ahtonen, 1994). At the same time, several opportunistic (bacteroids, peptostreptococci, clostridia, coliforms, staphylococci, *Gardnerella vaginalis*, *Streptococcus agalactiae seu* GBS, yeasts,

mycoplasmas *etc.*) and pathogenic microorganisms (*Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Listeria monocytogenes*, several viruses *etc.*) in the vagina are associated with several neonatal, puerperal and intraamniotic infections, preterm delivery, premature ruptures of membranes and spontaneous abortions (Gilbert *et al.*, 1995; Hillier *et al.*, 1995; McGregor *et al.*, 1995). However, the risk of emergence of these disorders is not solely dependent on the presence of the above-mentioned microorganisms in the genital tract. It requires further studies to clarify the microecological relations between different microorganisms and between macro- and microorganisms.

Since the longitudinal investigations of VMf during pregnancy have been very infrequent, it is not clear, if the altered VMf detected in the early phase of pregnancy disappears by the delivery or needs medical intervention and it is not known how several treatment regimens (antibacterial, hormonal) during pregnancy may influence the VMf. Nor it is quite clear which VMf is more beneficial for the newborns' initial colonization with microorganisms. It has been found that even a single-dose intrapartum administration of ampicillin which is generally used to prevent neonatal contamination by GBS, disturbs the neonatal colonization with the normal microflora in the same way as caesarean section does (Ahtonen *et al.*, 1993).

The present study focuses on investigating the VMf during pregnancy. We have tried to work out a new approach, which would take into account microbial interactions in the microecology of the vagina. By this means we have sought to answer the following questions:

— what must the composition of the VMf of a healthy woman be like under different physiological conditions and for safe delivery,
— what is the role of the VMf in case of certain pregnancy-related pathology,
— how the mother's VMf influences the initial microbial contamination of her newborn.

2. REVIEW OF LITERATURE

2.1. VAGINAL MICROFLORA STUDIES

2.1.1. History and state of the art

The vaginal microflora has been a subject to scrutiny since the late 1800s. The first extensive study was published as early as in 1892 by Döderlein who inspected stained smears and found that a healthy vagina principally harboured a single species, Döderlein's bacillus, which today is known as lactobacillus. Cultures of vaginal secretions soon revealed considerably more varied flora than this, including coliforms, diphtheroids, aerobic gram-positive cocci, and other microorganisms. Anaerobic bacteria were spotted in the normal vagina as early as in 1928 by Harris and Brown, but only at the beginning of the '70s did Gorbach *et al.* (1973) emphasize that anaerobes were also an important component of the normal genital flora. More recent studies have reported an average of 4-9 different microbes per vaginal sample (Stahl and Hill, 1986).

The main reason for numerous VMf studies has been the question about the relation between the microflora and its host — is the VMf influenced by several diseases or could the microflora cause them.

For a number of years VMf studies were **qualitative** or prevalence studies, i.e. various microorganisms were isolated, but no attempt was made to determine their amounts. In these investigations a wide variety of vaginal microorganisms were described (Tashjian *et al.*, 1976; Osborne *et al*, 1979; Hill, 1980). The species detected in the vagina vary from woman to woman and up to 100 different species and species-groups have been identified. Among the most frequently found microorganisms were lactobacilli, diphtheroids, staphylococci, streptococci, mycoplasmas (Table 1).

These prevalence studies could not describe the VMf in its full complexity. It became clear that the mere occurrence of any particular microorganisms in the microflora might not play the main role in the functions of the microflora. There appeared the necessity for a more advanced approach which could solve the problems from the microecological point of view.

For this purpose, several **quantitative** studies (Table 2) have been performed (Levison *et al.*, 1977; Bartlett and Onderdonk 1977; Lindner *et al.*, 1978; Bartlett and Polk, 1984; Cook *et al.*, 1984; Masfari *et al.*, 1986; Onderdonk *et al.*, 1986; Wilks and Tabaqchali, 1987; Hammann *et al.*, 1987; Sokolova *et al.*, 1988). These studies have yielded a wide variety of results (Larsen and Galask, 1980; Stahl and Hill, 1986; Redondo-Lopez *et al.*, 1990) and some contradictory data have been presented. For example, different investigators have found different species of lactobacilli (Onderdonk and Wissemann 1993); bacteroids have been found rarely (Ohm and Galask, 1975; Onderdonk and

Wissemann 1993) or else as the most common anaerobe in the vagina (Hammann *et al.*, 1987); *E. coli* has usually been considered rare and not numerous (Lindner *et al.*, 1978; Cook *et al.*, 1984), whereas some studies find it to be the most frequent aerobe (Horvath and Fazekas, 1989).

Table 1

Qualitative studies of the vaginal microflora: Incidence of particular microorganisms

Microorganisms	Frequency of occurrence (%) by		
	Hill 1980	Tashjian 1976	Osborne 1979
AEROBES			
Lactobacilli	45	88	58
Diphtheroids	55	72	14
G. vaginalis	58	22	2
Staphylococci	47	2	34
S. aureus	1	8	32
Group B streptococci	6	18	18
Group D streptococci	38	32	
Non-hemol. streptococci	25		14
α-hemol. streptococci	28	36	28
Gram-negative rods	36	26	20
Yeasts	12	30	16
Mycoplasma		12	22
Ureaplasma		58	28
ANAEROBES			
Lactobacilli	43		12
Eubacteria	4		0
Bifidobacteria	8		10
Propionibacteria	5		
Clostridia	11	4	8
Peptococci	>24		14
Peptostreptococci	32		32
Bacteroids	>13	32	50
Fusobacteria	19		10
Gram-negative cocci	22	2	10

Table 2

Quantitative studies of the vaginal microflora: Incidence and count of particular microorganisms

Micro-organisms	Frequency of occurrence (%) and count (log CFU/g or log CFU/ml) by					
	Levison 1977	Bartlett 1977	Lindner 1978	Bartlett 1984	Cook 1984	Wilks 1984
AEROBES						
Lactobacilli	71(7.5)	50(8.7)	80(8.6)	58(8.1)	87(7.2)	90(7.4)
Staphylococci	0(5.6)	41(7.5)	10(8.0)	52(5.8)	62(4.0)	60(7.2)
S. aureus		5(6.8)		3(6.1)		5(5.9)
Corynebacteria	0(5.8)	31(7.2)	8(8.1)	46(6.1)	39(4.2)	60(7.0)
G. vaginalis	43(7.8)				17(7.7)	20(6.2)
Streptococci	14(5.6)	59(6.8)	10(7.7)	48(6.5)	25(4.9)	20(7.3)
Enterococci	0(5.0)	27(7.0)		14(6.3)		15(6.9)
Mycoplasma						14(3.7)
Yeasts			16(7.2)	13(5.8)	14(4.2)	
Gram-neg.rods		9(6.4)	6(6.6)	8(5.6)	4(4.5)	15(5.1)
ANAEROBES						
Peptococci	57(7.0)	27(7.6)	4(7.8)	80(7.8)		10(6.5)
Peptostrepto-cocci	14(6.8)	14(8.3)		28(7.7)		15(6.7)
Lactobacilli	29(7.3)	45(8.2)		43(7.8)		60(7.6)
Eubacteria	0(6.2)	36(8.4)		20(8.3)	8(6.9)	10(8.0)
Bacteroids	43(7.5)	5(8.5)	4(8.3)	34(7.7)	14(5.8)	5(7.7)
Fusobacteria	0(7.4)	23(8.5)		9(7.9)	2(5.0)	10(7.7)
Veillonella	29(6.0)	9(7.6)				
Propioni-bacteria	0(6.7)	14(8.6)			4(5.2)	
Bifidobacteria	14(7.9)	5(8.6)			6(6.3)	15(8.1)
Clostridia		18(8.5)		7(6.9)		5(6.5)
Ureaplasma					54(5.0)	

There is an agreement in the average concentration of bacteria (10^8-10^9 colony forming units per gram or ml of secretions), the frequent predominance of aerobic lactobacilli in fertile and pregnant women, and the large numbers of coryneforms, anaerobic gram-positive cocci, anaerobic gram-negative bacilli and anaerobic gram-positive bacilli being often present. Anaerobic microbes frequently outnumber aerobic microorganisms. As the rule, cooperative dominance of several species is observed in a particular vaginal flora, and no particular species has been shown consistently predominate when present.

However, research describing the **microfloral ecosystem** of the vagina as a whole is sparse (Overman, 1993). Recently Ross *et al.* (1994; 1995) have proposed an interesting ecological approach for the assessment of VMf using statistical and *in vitro* models. Also the study of Lee *et al.* (1995) who have investigated healthy vaginal microbiota using cluster analysis and divided them into three subgroups, seems to be a promising one.

2.1.2. Critical assessment

As could be seen from previous studies, with these data it is almost impossible to evaluate a particular vaginal sample for diagnosing abnormality since there is still no full agreement as to what the VMf of a healthy woman should be like. We could presume some possible reasons for different and contradictory results:

1) different sampling sites within the vagina (the upper or lower part, the lumen or mucosa, *etc.*);
2) different laboratory techniques;
3) difference in external factors controlling the microflora within sampling time;
4) the vaginal microecosystem like any other one is dynamic, but studies of the normal microflora tend to be adynamic.

Thus, there is a particular need for data about changes in the VMf which depend on the menstrual cycle, pregnancy, age, *etc.* These could be gained only through additional longitudinal studies of individuals making it possible to predict if certain changes are temporary and disappear without treatment or need medical interference.

Consequently, when investigating the VMf of either a particular woman or a group, attention must be paid to 1) incidence, 2) quantity, and 3) interactions between the microorganisms in the microflora in dynamics.

2.2. SIGNIFICANCE OF THE VAGINAL MICROFLORA

It is difficult to overestimate the significance of the indigenous VMf since it can have both positive and negative effects on the health of women and their newborns.

2.2.1. Importance for the woman

On the positive side is the colonization resistance — protection provided by the normal flora against the invasion of overt pathogens or against overgrowth and predominance by potentially pathogenic species among the normal flora. Both situations can result in a disease. The relatively acid environment, low Eh, increased levels of short-chain fatty acids and other components of the vaginal fluid (mucus, lysozyme, lactoferrin, zinc, fibronectin, secretory IgA) contribute to this important function of microflora (Redondo-Lopez *et al.*, 1990; Onderdonk and Wissemann, 1993; McNicol *et al.*, 1994).

On the negative side can be considered the potential pathogenicity of members of the normal flora which under certain conditions could result in several diseases (Stahl and Hill, 1986).

2.2.2. Importance for the newborn

2.2.2.1. General considerations

The vaginal flora seems to become progressively more benign during pregnancy until at birth the flora is predominantly composed of organisms that do not pose a significant hazard to the fetus passing through the heavily colonized birth canal (Larsen and Galask, 1980; Mikelsaar *et al.*, 1989). This passage is extremely important for the newborn's initial microbial contamination which serves as a basis for further development of its individual microflora (Ross and Needham, 1980; Rotimi and Duerden, 1981; Bennet, 1987; Mikelsaar *et al.*, 1989; Torres-Alipi *et al.*, 1990; Ahtonen *et al.*, 1993; Jarvis, 1996) and helps to avoid colonization of the neonate with more dangerous microbes from other sources (Usacheva *et al.*, 1988; Belokrysenko, 1990; Keyworth *et al.*, 1990; Hall *et al.*, 1990).

On the other hand, contamination of the newborn during delivery or antenatally with opportunistic (Group B streptococci, genital mycoplasmas, coliforms, enterococci, *G. vaginalis*, staphylococci, *C. perfringens*, bacteroids) or pathogenic microorganisms (*Listeria monocytogenes, Toxoplasma gondii, Chlamydia trachomatis*, viruses, *Haemophilus influenzae*) may lead to the early

onset sepsis or other neonatal diseases due to immaturity of the neonates's immune system (Bennet, 1987; Dwyer and Cunningham, 1993; Ollikainen *et al.*, 1993; Ault, 1994; Berger *et al.*, 1995; Daniel *et al.*, 1995; Yancey *et al.*, 1996).

Some investigations have followed mother-baby transmission of particular single microorganisms (Carlsson and Gothefors, 1975; Hall *et al.*, 1990; Tannock *et al.*, 1990; Vonweizsacker *et al.*, 1995; Adriaanse *et al.*, 1995; Harvey *et al.*, 1995). However, there are quite few comparative studies focusing on mothers' delivery tract and their newborns' whole microflora immediately after birth (Brook *et al.*, 1979; Torres-Alipi *et al.*, 1990; Scheven and Ziegler, 1990; Ahtonen *et al.*, 1993), and only very few quantitative studies can be found (Mikelsaar *et al.*, 1989).

2.2.2.2. Importance of *Streptococcus agalactiae* (GBS)

Streptococcus agalactiae (GBS) is an important cause of neonatal sepsis, pneumonia and meningitis (Adams *et al.*, 1993; Parea *et al.*, 1994; Koutouby and Halibullah, 1995; Gilbert *et al.*, 1995; Jafari *et al.*, 1995). Two different expressions of the GBS disease are reported to occur — the early onset disease and the late onset disease. The early onset disease begins within 24 h of birth and is probably due to the vertical mother-to-infant mode of transmission while late onset disease is due to nosocomial acquisition of GBS. It has been shown that in different countries some 5–40% of fertile women may be the carriers of GBS and about 50% of their offspring will be colonized. Only 1–2% of these babies develop disease (Gibbs *et al.*, 1992) whose mortality is approximately 15% (Parea *et al.*, 1994). The risk factors for mother-to-infant transmission of GBS are believed to be premature labor, intrapartum fever, prolonged rupture of membranes, multiple births and high density of GBS in delivery tract (Boyer *et al.*, 1983; Parea *et al.*, 1994). Antenatal contamination of baby is possible, too, since it is known that GBS is able to cross also intact amniotic membranes (Katz and Bowes, 1988; Carstensen *et al.*, 1988). The risk factors for the development of disease in colonized newborn are thought to be the low titre of antibodies in the mother (Föster and Borkhardt, 1988; Helmig *et al.*, 1993), certain types of GBS (Föster and Borkhardt, 1988) and the number of receptors in the newborn (Broughton and Baker, 1983). Since the occurrence rate has been found to be quite different in different study populations and countries the data about Estonian women will be important for both gynaecologists and neonatologists.

2.2.2.3. Importance of *Clostridium difficile*

C. difficile is an important cause of antibiotic-associated diarrhoea and pseudomembranous colitis. It can be isolated from 2–3% of healthy adults but from 10–95% of neonates. The harbouring of *C. difficile* occurs mainly during the first 8 months of life, after the second year of life it decreases to the adults' level (Tullus *et al.*, 1989; Miyazaki *et al.*, 1992; Cherkasskaia *et al.*, 1992; Collignon *et al.*, 1993; Knoop *et al.*, 1993). Despite of the high occurrence rate of *C. difficile* in newborns' gastrointestinal tract, the newborns have *C. difficile*-associated diarrhoea very infrequently. This has been associated with the occurrence of only spores in the newborns' gastrointestinal tract (Miyazaki *et al.*, 1992), the absence of toxin binding sites (Eglow *et al.*, 1992) and the presence of mainly nontoxigenic strains in newborns (Kato *et al.*, 1994). However, Lehtonen *et al.* (1994) have found the colicky infants to be more frequently colonized with *C. difficile*. The reason and the source of so frequent colonization of the newborns by *C. difficile* is not clear. Antibacterial treatment which is an important risk factor in adults, does not influence or even delays the intestinal colonization by *C. difficile* in newborns (Holton *et al.*, 1989). It has been suggested that the source could be the hospital environment and the hands of the personnel (Al-Jumaili *et al.*, 1984; Bacon *et al.*, 1988) since caesarean section and a long stay in the nursery increase the colonization rate (Bacon *et al.*, 1988; Ahtonen 1994). The source could also be the mothers' genital tract. Tabaqchali *et al.* (1984) have found *C. difficile* in the vagina in 8...18% of women, but Al-Jumaili *et al.* (1984) and Manso *et al.* (1986) in none. At the same time, many of their children were colonized by *C. difficile*.

2.3. FACTORS CONTROLLING THE VAGINAL MICROFLORA

The vaginal microecosystem is an open system which can be influenced by **endogenous** and **exogenous** factors. These factors may cause fluctuations in the system, but usually the endogenous regulatory mechanisms keep it in a relatively stable balance.

2.3.1. Endogenous factors

The major factor controlling the microbial types and population levels in the vagina is generally believed to be the hormonal status of a woman (Molander *et al.*, 1990; Redondo-Lopez *et al.*, 1990; Onderdonk and Wissemann, 1993).

This varies depending on age, the menstrual cycle and pregnancy, and the VMf has also been found to vary depending on these changes.

The VMf is undoubtedly influenced by the composition of vaginal secretions which include contribution from vulvar secretions, Bartholin's and Skene's glands, transsudates from the vaginal wall, exfoliated cells, cervical mucus and endometrial and oviductal fluids. The following substances are commonly present: NaCl, potassium, sulphates, vitamins, metal ions, mucins, proteins, immunoglobulins, enzymes (glucosidase, amylase, antitrypsin), complex carbohydrates, lipids and fatty acids (Redondo-Lopez *et al.*, 1990; Onderdonk and Wissemann, 1993).

The redox potential, presence of H_2O_2, CO_2 and blood, inflammatory response, anatomic ultrastructural changes, and many other factors seem also to be important (Larsen and Galask, 1980; Stahl and Hill, 1986; Knothe *et al.*, 1987; Neumann, 1988; Redondo-Lopez *et al.*, 1990).

2.3.2. Exogenous factors

Contraceptive devices, sanitary methods, surgery in the genital tract, antimicrobial or immunosuppressive treatment, extragenital diseases, coitus, blood group, and many other factors may influence the vaginal microecosystem (Mardh, 1991; Milsom *et al.*, 1993; Dekker, 1993; Navas *et al.*, 1993; Hooton *et al.*, 1994).

Antimicrobial treatment may increase the risk of infection or disease through the disruption of the protective microflora barrier (Sjöberg *et al.*, 1990; Winberg *et al.*, 1993; Ross *et al.*, 1995). The influence of antibiotics on the VMf is apparently mainly connected with the disturbance of vaginal lactobacilli. The administration of antimicrobials which have no great effect on lactobacilli, may therefore be advantageous to the host. However, since the susceptibility of vaginal lactobacilli has been insufficiently studied, it is difficult to follow this suggestion.

The data at present available suggest that most strains of lactobacilli are susceptible to penicillins and most other beta-lactam agents and also to clindamycin and chloramphenicol (Bantar *et al.*, 1991; Koneman *et al.*, 1992) but resistant to sulphonamides and fosfomycin (Cooper *et al.*, 1990; Hamilton *et al.*, 1994). Susceptibility to cephalosporins and quinolones varies (Koneman *et al.*, 1992; Hamilton *et al.*, 1994; Herra *et al.*, 1995). Different investigators have found some strains of lactobacilli to be resistant to vancomycin (Bayer *et al.*, 1978; Bantar *et al.*, 1991; Mackey *et al.*, 1993). Lenzner *et al.* (1980) have found that susceptibility to vancomycin can be determined by species of lactobacilli: *L. helveticus, L. delbrueckii ssp. lactis* and *L. acidophilus* were susceptible to vancomycin, but *L. delbrueckii ssp. delbrueckii, L. salivarius* and

all strains of facultatively and obligatorily heterofermentative lactobacilli showed resistance to it. In vagina there could be found many of the above-mentioned species (Redondo-Lopez *et al.*, 1990; Onderdonk and Wissemann, 1993).

At the same time, the increasing resistance of microorganisms which probably is the result of the extensive use of antibiotics has become a worldwide problem (Cassell, 1995; Salyers and Shoemaker, 1995). Some antibiotics, like third-generation cephalosporins and quinolones have been in wide use for a comparatively shorter period in Estonia than in the more advanced western countries (Kiivet, 1991; Reports of the State Agency of Medicines, 1995). Thus, it can be supposed that indigenous microorganisms, including VMf, isolated from the different countries may harbour different susceptibility patterns, however, no studies have confirmed it.

2.3.3. Mechanisms of influence

The mechanisms by which all these factors influence the microflora are not clearly understood. The hormonal status, vaginal acidity (pH) and glycogen content have been most thoroughly studied and found to be related to vaginal lactobacilli. Estrogen stimulates the production and deposition of glycogen in vaginal epithelial cells. Bacterial enzymes degrade it into glucose which will be reduced to lactic acid. This process is thought to contribute to the acid environment of the vagina (pH 4-5), which supports the survival and growth of acidophilic microorganisms (Onderdonk and Wissemann, 1993). However, the question remains, if the low pH is the reason or the effect of vaginal *Lactobacillus* colonization (Redondo-Lopez *et al.*, 1990; Larsen, 1993).

It has been proposed that the ability of lactobacilli to adhere to vaginal epithelial cells is a major feature of the vaginal environment (Onderdonk and Wissemann, 1993). This adherence is influenced by several factors: coinfluence of several hormones (Thadepalli *et al.*, 1982; Brilene *et al.*, 1989; Brilene, 1990); vaginal pH (Larsen, 1993; Nagy *et al.*, 1995); presence of nutrients such as glycogen, presence of other microorganisms (Overman, 1993); properties of lactobacilli (Horowitz *et al.*, 1994). The presence of lactobacilli in vagina seemingly helps to create an acidic environment, they can also produce hydrogen peroxide and other antimicrobial substances, compete with the other microorganisms for adherence, and stimulate the immune system (Redondo-Lopez *et al.*, 1990).

However, little is known about the establishment of stable relations between different coexisting microorganisms — the microecological relations, and the mechanisms influencing these relations.

2.4. VAGINAL MICROFLORA DURING PREGNANCY

The comparatively few and quite old studies (De Louvois *et al.*, 1975; Tashjian *et al.*, 1976; Lindner *et al.*, 1978; Zai *et al.*, 1986; Mendling, 1987; Walss-Rodriguez *et al.*, 1988) have shown that in the VMf during pregnancy the anaerobic species decline and lactobacilli become increasingly predominant. Also yeasts can be found more frequently than in nonpregnant women. The possible reason for these changes is the altered hormonal state, increased pH, and increased glycogen content of the vaginal epithelium (Galask, 1988). Similarly to nonpregnant women, bacterial vaginosis (BV) can be found during pregnancy in 10–26% of women (Cristiano *et al.*, 1989; Thomason *et al.*, 1991). However, only a few investigators have tested pregnant women for BV repeatedly (Platz-Christensen, 1993; Riduan, 1993; McDonald *et al.*, 1994).

Investigations of the VMf during pregnancy have been quite sporadic, and mostly qualitative. We found no studies testing women's VMf repeatedly during pregnancy. However, we found one study about dynamics of the cervical microflora during pregnancy (Goplerud *et al.*, 1976).

2.5. PATHOLOGICAL CHANGES IN THE VAGINAL MICROFLORA

Opportunistic and pathogenic microorganisms may cause several genital tract disorders. It is appropriate to consider what characteristics of the microorganisms enable them to harm the host and what properties of the microenvironment support these capabilities.

2.5.1. Pregnancy-related pathology

2.5.1.1. Perinatal, puerperal and intraamniotic infections

The microbes connected with perinatal, puerperal and intraamniotic infections are often found to originate from the indigenous VMf (Coultrip *et al.*, 1991; Gauthier *et al.*, 1991; Gibbs, 1993; Newton, 1993; Krohn *et al.*, 1995). However, the presence of certain microorganisms in a patient's genital tract alone does not allow the physician to predict the relative risk of infection. One possible approach is to study the microecological balance in the vagina by determining the quantitative relations between different microorganisms since it has been shown that the microecological status is a good marker of health and disease (Mikelsaar, 1992).

2.5.1.2. Premature labor, preterm delivery and premature rupture of membranes (PROM)

Premature labor, preterm delivery and PROM are often connected with infectious agents (Andrews *et al.*, 1995) — the opportunistic members of the normal VMf (Dodson and Fortunato, 1988; Toth *et al.*, 1988; Romero *et al.*, 1989; Olah and Gee, 1992; McGregor *et al.*, 1995) but also by genital pathogens - BV-related microorganisms like *G. vaginalis*, bacteroids, peptostreptococci, mycoplasmas, *Mobiluncus sp.* (Eschenbach, 1993; Hill, 1993; McDonald *et al.*, 1994a; Hillier *et al.*, 1995a,b), *Chlamydia sp.* (Martius *et al.*, 1988; Dodson and Fortunato, 1988; Pastorek, 1989), GBS (Matorras *et al.*, 1989; McDonald *et al.*, 1989; Hillier *et al.*, 1991), *N. gonorrhoeae* (Ekwo *et al.*, 1993). It is known that normal delivery is induced by phospholipase A which originates from amnion or chorion (Bejar *et al.*, 1981). Phospholipase activity is known to be characteristic of many genital microorganisms (McGregor *et al.*, 1991), including BV connected microbes (Kurki *et al.*, 1992), and these microbes may also produce proteases, neuraminidase, mucinase, collagenase, that may predispose cervical ripening, preterm delivery or PROM (McGregor *et al.*, 1995).

2.5.1.3. Spontaneous abortion

Although there can be found numerous studies concerning the influence of the VMf on the above-described delivery-related pathology, the number of studies about the VMf in an early spontaneous abortion or threatened abortion (TAb) is far from sufficient. Spontaneous abortion may be caused by fetal, maternal or external factors, and in many cases, no specific etiology can ever be identified (McBride, 1991). The early TAb has mainly been connected with genetic (Antipenko and Alekseyenko, 1992; Delporto *et al.*, 1993; Tulppala *et al.*, 1993), immunological (Pratt *et al.*, 1993; Tulppala *et al.*, 1993), and hormonal changes (Watson *et al.*, 1993; Harrison, 1993), stress (Amore *et al.*, 1992), smoking (Floyd *et al.*, 1993), placenta praevia (Taylor *et al.*, 1993) or other factors (Fox, 1993; Seller *et al.*, 1993).

Relatively infrequently have abortions been associated with infection (Table 3; Daugaard *et al.*, 1988; Leiberman *et al.*, 1989; Buzoni-Gatel *et al.*, 1990; Quentin *et al.*, 1993; Clark *et al.*, 1993; Heisterberg, 1993; Jensen and Schonheyder, 1993) and few of these studies have dealt with the first trimester pregnancy loss. Also bacterial vaginosis has been connected with spontaneous abortion (Hay *et al.*, 1994; Mcgregor *et al.*, 1995). As concerns HIV infection, it has been shown that asymptomatic women have no increased rate of

spontaneous abortion, but associated chronic viral or protozoal infections may increase the risk. Many different mechanisms have been proposed to explain an infectious case of abortion (Table 3). Toxic metabolic byproducts, endo- or exotoxin, or cytokines could have an adverse effect on the uterus or the fetoplacental unit. Alternately, fetus, placenta or amnion could be directly infected. Endometritis could interfere with implantation. The data of the studies described above suggest that infection is an occasional cause of spontaneous abortion. Probable factors that may increase the risk of abortion due to infections are the exposure during early gestation, placental involvement, immunocompromised host (Summers, 1994).

It is not clear, whether the changes in VMf concerning the indigenous microorganisms are the cause of abortion or merely indicators of a changed vaginal environment due to several influencing factors. Nor is it clear, whether such early altered VMf persists up to the end of pregnancy and influences the course of delivery and the newborn or not.

Table 3

Postulated mechanisms of spontaneous abortion for selected pathogens

Mechanisms	Microbes/diseases
Fetotoxicity/ embryopathy	Rubella, parvovirus B19, coxsackie B, varicella-zoster, chronic cytomegalovirus, herpes simplex, syphilis, Lyme disease
Placental congestion	Malaria
Endometritis/ endocervicitis	Chlamydia, *Ureaplasma urealyticum, Mycoplasma hominis*
Amnionitis or infected IUD	Various opportunistic G+ and G– bacteria, *Listeria monocytogenes*

2.5.2. Microecological disorders

In some cases the regulatory factors cannot guarantee a balanced VMf and such situations may result in certain microecological diseases such as BV, vaginal candidiasis and also recently described cytolytic vaginosis (Cibley and Cibley, 1991) and vaginal lactobacillosis (Horowitz *et al*, 1994). During pregnancy the BV and candidiasis deserve more attention.

2.5.2.1. Bacterial vaginosis

One of the most widespread and comparatively well investigated pathological conditions of the VMf is bacterial vaginosis (BV). Despite numerous studies since the first description of it by Gardner and Dukes (1955) its nature has still remained unclear and it is called "an ecologic mystery" (Sobel, 1989). BV is an imbalance of vaginal microecosystem in which microaerophilic lactobacilli are absent from the vagina, while there exists codominance of *Gardnerella vaginalis, Mobiluncus sp., Mycoplasma hominis*, and some anaerobic microorganisms. This potentially pathogenic flora and conditions that allow their overgrowth coexist in case of BV (Mardh, 1991; Spiegel, 1991; Mead, 1993; Larsen, 1993; Hillier, 1993; Kira, 1994; Holst *et al.*, 1994; Mikamo *et al.*, 1996). But it is not clear what is of primary importance — the appearance of infectious microorganisms or the disappearance of lactobacilli (Briselden and Hillier, 1990; Fredricsson *et al.*, 1992; Overman, 1993; Kasprowicz and Bialecka, 1993). Contradictory opinions have been expressed about the role of temporary factors like the intrauterine device, sexual activity, hormonal state of the organism, change of vaginal pH (Bump and Buesching, 1988; Thomason, 1991; Roy, 1991; Biswas, 1993; Levett, 1995). Rectum has been found to be a reservoir of BV-related microbes (Hallen *et al.*, 1988; Holst, 1990) and lactobacilli different from those of the normal vagina (Nagy *et al.*, 1992). BV is often asymptomatic (Amsel *et al.*, 1983; West *et al.*, 1988), but not fully safe because of possible pregnancy-related complications, the upper genital tract infections and production of cancerogenic nitrosamines (Nugent *et al.*, 1991; Kurki *et al.*, 1992; Eschenbach, 1993; McGregor *et al.*, 1995; Sweet, 1995).

2.5.2.2. Vaginal candidiasis

Another widespread disorder — vaginal candidiasis — is also an "ecologic" disease because there is a strong correlation of its signs and symptoms with the count of yeast cells in the vagina (Odds *et al.*, 1988), synergistic and antagonistic influence on many other vaginal bacteria (Robertson, 1988), strong

relationship with hormonal state of the host (Spinillo *et al.*, 1995; Geiger *et al.*, 1995) and frequent development after the normal flora has been destroyed by antibacterial treatment (Roy, 1994). The most common yeast species found in the vagina is reported to be *Candida albicans*, sometimes *C. glabrata, C. tropicalis* or other species can be isolated (Sullivan and Smith, 1993; Goode *et al.*, 1994). Vaginal candidiasis has more often been found during pregnancy than in nonpregnant women, the one possible reason being the suppression of neutrophils' activity by increased level of progesterone (Nohmi *et al.*, 1995; Levett, 1995).

* * *

Consequently, there are a lot of unsolved problems yet —

— what must a healthy woman's VMf be composed of under different physiological conditions to guarantee health for the woman and her newborn,
— do the regulatory mechanisms always ensure the maintenance or re-establishment of a beneficial composition of the VMf,
— how is the VMf associated with pregnancy-related pathology,
— how the VMf influences the initial microbial contamination of the newborn.

3. OBJECTIVES OF THE INVESTIGATION

I. Elaboration of methods for evaluating the vaginal microflora in pregnancy.

II. Application of the elaborated methods:

1. for investigating the dynamics of the vaginal microflora in the course of pregnancy and to the study of some factors influencing it;

2. for comparison of the vaginal microflora in the normal course of pregnancy and in case of an early threatened abortion;

3. for comparison of the vaginal microflora in the normal and adverse pregnancy outcome.

III. Study of the putative transmission from mother to newborn of:

1. normal microflora;

2. some opportunistic pathogens.

4. MATERIAL AND METHODS

Altogether 138 women and 34 newborns were involved in the study. 391 analyses of the vaginal microflora and 22 analyses of the rectal microflora from pregnant women and 34 analyses of the external ear canal microflora from newborns were performed.

4.1. SUBJECTS AND COLLECTION OF MATERIAL

4.1.1. Pregnant women

Quantitative bacteriological studies of the VMf were carried out on 69 pregnant women admitted to the Tartu Maternity Clinic (Table 4). **Group I** consisted of 23 pregnant women asymptomatic at arrival, inclusion criteria for them were: presenting before the 17th week of gestation, the first expected delivery, body weight (kg) before pregnancy was less than body length (cm) – 100 – 10%. **Group II** consisted of 19 and **Group III** — of 27 women both consecutive, they were considered eligible for the study if they were hospitalized due to a TAb before the 12th week of gestation. We considered as TAb multiple painful uterine contractions with or without vaginal bleeding which did not lead to abortion due to the treatment the women received.

All the women of Groups I and II were examined 4–7 times during pregnancy, the total number of investigations before delivery being 234 (117 in both groups). The periods of sampling were: ≤10 weeks, 11th–16th, 17th–22nd, 24th–26th, 28th–30th, 32nd–34th and 36–38th weeks of gestation. The women of Group III were examined twice during pregnancy: at the time of threatened abortion and in the 32nd week of pregnancy, the total number of estimations being 54.

The data about the course of delivery were analyzed. All women of Group II, 25 women of Group III and 21 women of Group I delivered live babies, one delivered a foetus mortus with multiple malformations. We lack information about the results of delivery of some women: one of Group I and two of Group III. One woman of Group I, four women of Group II and two women of Group III delivered by caesarean section. Premature labor, preterm delivery and/or premature rupture of membranes were observed in 11 women of Group I, in 11 women of Group II and in 7 women of Group III.

To study more precisely **some genera of indigenous and opportunistic vaginal and rectal microorganisms** and some of their characteristics during pregnancy, three groups of consecutive pregnant women (Groups IV–VI) were included. Vaginal and rectal samples were taken from women of **Group IV** and vaginal samples from women of **Groups V and VI** at their arrival for the regular control during pregnancy. 22 consecutive pregnant women were investigated once during the third trimester of pregnancy (Group IV) and 30 consecutive women were studied once during pregnancy (Group V) at Tartu Maternity Clinic. Group VI consisted of 17 consecutive pregnant women examined once at Indiana University Hospital (Indiana, USA).

4.1.2. Mother-baby pairs

Out of 42 mothers of Groups I and II we succeeded in 34 cases to follow the putative transfer of microflora to their newborn during delivery (Table 5). 19 mother-newborn pairs were examined immediately after birthgiving (Group A), 4 pairs were investigated between 1 and 12 hours after delivery (Group B), and 11 pairs — within 2 to 8 days after delivery (Group C). For several reasons (caesarean section or some technical problems) we failed to study the remaining 8 pairs.

4.1.3. Collection and transport of the specimens

For the women of Groups I–VI, the specimens were taken from the lateral side of the internal third of the vagina, for the women of Group IV also from the *ampulla recti*. For the newborns, the samples from external ear canal were investigated.

In order to maintain the viability of fastidious microorganisms, in case of Groups I, II and III the blood-thioglycollate-agar-coated cotton-wool swabs were used (Mikelsaar *et al.*, 1989). The swabs were put into tubes containing carbon dioxide and sealed with rubber stoppers. The specimens were sent to the laboratory within 2 hours of collection. In case of Groups IV, V and VI the Stuart transport medium for anaerobes (Baron and Finegold, 1990) was used.

Table 4

Clinical data of pregnant women investigated

Group	No of women	No of investigations	Age	No of prev. pregnancies	Number of women with		
			range (mean)		AMT *	HT **	APO ***
Group I	23	117	10–29 (22.9)	0–2 (0.3)	9	0	11
Group II	19	117	20–35 (26.7)	0–5 (2.2)	8	19	11
Group III	27	54	18–33 (25.2)	0–7 (2.4)	5	27	7
Group IV	22	44	18–30 (24.1)	0–6 (1.5)	–	–	–
Group V	30	30	17–37 (23.4)	0–2 (0.9)	–	–	–
Group VI	17	17	–	–	–	–	–

* Antimicrobial treatment: Nitrofurans for urinary tract infections (4 women of Group I; 4 women of Group II; 3 women of Group III), Metronidazole for trichomoniasis (1;1;0), Clotrimazole (3;0;0) or Nystatini (0;1;1) for vaginal candidiasis, Sulphonamides (2;3;1), Ampicillini (0;1;1) or Oxacillini (0;1;1) for respiratory tract infections. All the drugs were used as short courses during the 2nd and 3rd trimester of pregnancy.

** Hormonal treatment: *Turinal* (Gedeon Richter, Hungary), contains 5 mg allyloestrenoli. The hormonal therapy (5–15 mg daily) was started at arrival and stopped before the 20th week of gestation.

*** Adverse pregnancy outcome: Premature labor (2 cases in Group I; 1 case in Group II; 4 cases in Group III), preterm delivery (1;2;0), premature rupture of membranes (5;8;3), combination of two adverse outcomes (3;0;0).

Table 5

The investigated mother-baby pairs

Mother-baby pairs	No of pairs, during pregnancy belonging to		
	total	Group I	Group I
Immediately after delivery (Group A)	**19**	12	7
1 to 12 hours after delivery (Group B)	**4**	2	2
2 to 8 days after delivery (Group C)	**11**	7	4

4.2. MICROBIOLOGICAL METHODS

4.2.1. Preparation and cultivation of the specimens

To determine the **quantitative composition of the microflora**, the swabs of Groups I, II and III were shaken in 2 ml of pre-reduced phosphate buffer (pH 7.2) under a gentle stream of oxygen-free CO_2. Serial dilutions 10^{-1} ...10^{-5} of the bacterial suspension were prepared. The dilutions (0.01 ml) were then seeded into different media (Table 6): pre-reduced blood-thioglycollate-agar medium handled as modified roll-tubes for anaerobic microorganisms (Mikelsaar *et al.*, 1984), freshly prepared blood-agar with 5% of human blood for aerobic microorganisms, lactobacilli and streptococci selective MRS-4 agar (Lenzner *et al.*, 1984), Endo agar for coliforms, and Sabouraud agar for yeasts. By this method the detection level was $\geq$20 CFU/swab.

For the study of **some anaerobic and microaerophilic genera of microorganisms**, the vaginal and rectal swabs of Group IV were shaken in 2 ml of pre-reduced phosphate buffer (pH 7.2) under a gentle stream of oxygen-free CO_2. The suspension was streaked onto plates using calibrated loop, thus performing semiquantitative study. Fastidious Anaerobe Agar (F.A.A., LAB M) for anaerobic microorganisms, freshly prepared blood-agar with 5% human blood for aerobic microorganisms, egg yolk agar and Fastidious Anaerobe Agar with Fluorocult TSC-Agar Supplement (MERCK) for *Clostridium sp.*, and Cycloserine-Cefoxitin-Fructose-Agar (CCFA, Oxoid) for *C. difficile* were used

for both vaginal and faecal samples, and additionally MRS agar (MERCK) for lactobacilli was used for vaginal swabs.

The vaginal swabs of Group V were seeded directly onto MRS agar (MERCK) for isolation of lactobacilli and onto freshly prepared blood-agar with 5% of human blood for GBS. The swabs of Group VI were seeded directly onto Anaerobic Blood Agar (BBL) and Tomato juice agar (Koneman *et al.*, 1992) for isolation of lactobacilli.

Since we have used a few different methodologies in the course of the present study, we have **compared the results received by using the different media and methods**. In case of lactobacilli we used MRS-4 for studying the women of Groups I–III, and MRS produced by MERCK for studying the women of Groups IV and V. We did not find any great difference in these two media: we succeeded in isolating lactobacilli from 61.8% of samples of Groups I–III and from 63.5% of samples of Groups IV and V ($p=0.96$). In case of clostridia we used blood-thioglycollate-agar in Groups I–III and three selective media (Fastidious Anaerobe Agar with Fluorocult TSC-Agar Supplement (MERCK), Cycloserine-Cefoxitin-Fructose-Agar (CCFA, Oxoid) and egg yolk agar) in Group IV. We could notice some advantage of selective media: 10.1% versus 32%, however, the relative proportion of clostridia was quite low (< 1%) in most cases in all groups and the difference was statistically negligible ($p=0.052$).

Data about the methods of isolation of investigated microorganisms are presented in Table 6.

4.2.2. Identification of microorganisms

All the aerobes and facultative microorganisms were identified using standard methods up to the *genus* level. Group B streptococci were identified by CAMP-test with 5% of sheep blood (Baron and Finegold, 1990). The lactobacilli were identified on the basis of colonial and cellular morphology, negative catalase production and their ability to grow well on MRS agar (Lenzner *et al.*, 1984).

The anaerobes were identified up to the *familia* or *genus* level by colonial and cellular morphology and Gram stain reaction (Holdeman *et al.*, 1977; Stargel *et al.*, 1978; Mitsuoka, 1980). From the roll-tubes (Groups I–III) of the first, fourth and fifth dilution and from each agar plate (Group IV) the different types of colonies were counted. Out of every distinct type of colony 3–5 were characterized more precisely. The following cultural parameters of checked anaerobes were registered according to the following criteria: the colony size (pinpoint, small — 1–2 mm, medium, large — 4–5 mm), edges (regular, irregular), structure (homogenous, spotted), pigment (fluorescent black or not), emerging centre of colony, hemolyse (full or beta, greening or alpha, double

zone beta, no hemolyse), presence of gas and pitting phenomenon in thioglycollate-blood-agar. The presence of lipolysis and lecithinase was registered on egg yolk agar. After that, the aerotolerance of all the picked-up colonies of putative anaerobes on blood agar and MRS-2 (Lenzner *et al.*, 1973) was determined. Gram stained slides were made of each of the above-described colonies. By means of these slides the Gram stain reaction (positive, negative), microbial morphotype (cocci, bacteria, bacilli, vibrios), shape (uniform, branching, coryneforms, pleomorphic), size (tiny, small, large, coccobacteria), sharpened ends, structure (homogenous or vacuolisated e.g. swelling bodies, inclusions) and arrangement (single, paired, parallel, chained) were determined. As a result we could differentiate particular *familia* (bacteroids, anaerobic G+ cocci) or even *genus* (bifidobacteria, eubacteria, propionibacteria, actinomyces, clostridia, veillonella). *C. difficile* was identified on the basis of their characteristic colonial and cellular morphology on Cycloserine-Cefoxitin-Fructose-Agar (CCFA, Oxoid).

4.2.3. Counting of microorganisms

In each sample of Groups I–IV, the total count of microorganisms per swab was calculated, different microorganisms detected and their relative distribution (%) in the total number of microorganisms of vaginal or rectal microflora calculated.

4.2.4. Microscopic investigation of Gram-stained vaginal smears

The first dilution of the vaginal material of Groups I–III was used to make the Gram-stained smears. Then, the slides were examined using a combined method for evaluation of the vaginal microecosystem: the scoring system of Nugent and coworkers (1991) was applied to count microbial morphotypes, and registration of the "clue cells" was performed (Table 7).

Morphotypes of microorganisms were scored as the average number seen per 3 oil immersion fields: 0 — particular morphotype not present; 1+ — < 1 cells of this morphotype present; 2+ — 1 to 4 cells of this morphotype present; 3+ — 5 to 30 cells of this morphotype present; 4+ — 30 or more cells of this morphotype present. The criterion for bacterial vaginosis (BV) was a total score of 7 or higher; a score between 4 and 6 was considered intermediate, and a score between 0 and 3, classical. The presence of "clue cells" was registered to evaluate the BV-diagnosis (Thomason *et al.*, 1992).

Table 6

Media and incubation

Group	Microorganisms	Media	Incubation
ANAEROBES			
I–III	Bifidobacteria Eubacteria Anaerobic cocci Propionibacteria Actinomyces Bacteroids Clostridia Veillonella	Blood-thioglycollate-agar (roll-tubes)	37°C, oxygen-free CO_2-environment, 4–5 days
IV	*C. difficile*	Cycloserine-Cefoxitin-Fructose-Agar	37°C, environment generated by Gas Generator Envelopes for Production of an Anaerobic Atmosphere (BBL) (H_2 + 4–10% CO_2) 4–5 days
	Clostridium sp.	Fastidious Anaerobe Agar with Fluorocult TSC-Agar Supplement --- Egg Yolk Agar	
	Total count of anaerobes	Fastidious Anaerobe Agar	

Group	Microorganisms	Media	Incubation
FACULTATIVE ANAEROBES, AEROBES AND MICROAEROPHILES			
I–V	Staphylococci Streptococci Corynebacteria Bacilli Actinomyces	Blood-agar	37°C, 2–3 days
	Coliforms	Endo agar	
	Yeasts	Sabouraud agar	
I–III	Lactobacilli Streptococci	MRS-4 agar	37°C, 10% CO_2-environment, 2–3 days
IV–V	Lactobacilli	MRS agar (MERCK)	37°C, 10% CO_2-environment, 2–3 days
VI	Lactobacilli	Tomato juice agar, Anaerobic Blood Agar	37°C, 85% N_2 + 10% CO_2+ 5% H_2-environment, 2–3 days

4.2.5. Susceptibility testing of lactobacilli

The isolated strains of lactobacilli (n=36) from the women of Groups V and VI were tested for their susceptibility to 15 antibacterial agents using Kirby-Bauer disc diffusion method. Lactobacilli were suspended in thioglycollate broth to the density of 0.5 McFarland standard. The suspensions were inoculated onto agar plates using swabs and the BBL Sensi-Disc Susceptibility Test Discs were added. The plates were incubated at 37°C for 48 h. For Group V, we used Fastidious Anaerobe Agar with 5% of human blood (F.A.A., LAB M) in the environment of H_2 + 4–10% CO_2, for Group VI, we used Blood Agar Anaerobe (Carr Scaborough Microbiologicals Inc.) in the environment of 85% N_2 + 5% CO_2 + 10% H_2. As the breakpoint for every antibiotic was

considered the growth inhibition zone diameter between "resistant" and "intermediate" according to the manufacturer's instructions, conformed to the criteria of the National Committee for Clinical Laboratory Standards (1990).

4.3. STATISTICAL METHODS

The data were analyzed using the Wilcoxon rank test, Mann-Whitney rank sum test, *t*-test, Poisson rates, and regression and correlation analyses using program "Statgraphics".

Table 7

Methods for evaluation of the vaginal microecosystem on the basis of Gram-stained slides

Score	Large G+ rods (*Lacto-bacillus* morpho-type)	Small G- to G-var rods (*Gardnerella* and *Bacteroides* morpho-types)	Curved G-var rods (*Mobil-uncus* morpho-types)	Data of a particular sample		State of vaginal micro-flora
	I	II	III			
0	4+	0	0	Total score (I+II +III)	Clue cells (+/ -)	
1	3+	1+	1+ or 2+			
2	2+	2+	3+ or 4+			
3	1+	3+				
4	0	4+				
Examples:						
Sample 1	4+ (score 0)	1+ (score 1)	0 (score 0)	1	-	Classical
Sample 2	3+ (score 1)	3+ (score 3)	1+ (score 1)	5	-	Inter-mediate
Sample 3	0 (score 4)	4+ (score 4)	2+ (score 1)	9	+	Bacterial vaginosis

5. RESULTS AND DISCUSSION

5.1. DEVELOPMENT OF METHODS FOR THE EVALUATION OF THE STATE OF THE VAGINAL MICROFLORA DURING PREGNANCY (Group I)

(Paper I, II, IV)

We tried to elaborate some new methods and criteria for evaluating the VMf of pregnant women. For this purpose we investigated the consecutive women of Group I appearing for regular control during pregnancy.

5.1.1. Elaboration of methods for comparing different women (Group level)

(Paper I)

5.1.1.1. Counts of microorganisms

Various aerobic and anaerobic microorganisms were isolated from 115 of the 117 samples analyzed. No organisms were detected in 2 samples. The range of microorganisms throughout the course of pregnancy was 0–7.6 log CFU/swab, with a mean 6.7.

Anaerobic bacteria prevailed in 40 (34.8 %) samples, aerobic bacteria in 68 (59.1%) samples, and the counts of aerobes and anaerobes were nearly equal in 7 (6.1%) samples.

5.1.1.2. Occurrence of microorganisms

We isolated 19 different microorganisms from the investigated samples and none of them occurred in all the women. There were 0–9 different microbes in one sample (mean 3.6), lactobacilli being the most frequent microorganisms (Fig. 1). Streptococci, bacilli and corynebacteria were also often found.

5.1.1.3. Predominance pattern of the microflora

To get a better overview of the counts of different microorganisms colonizing the vagina, their relative proportion in the total count of microorganisms (%) was calculated. That made it possible to distinguish between the **prevailing and**

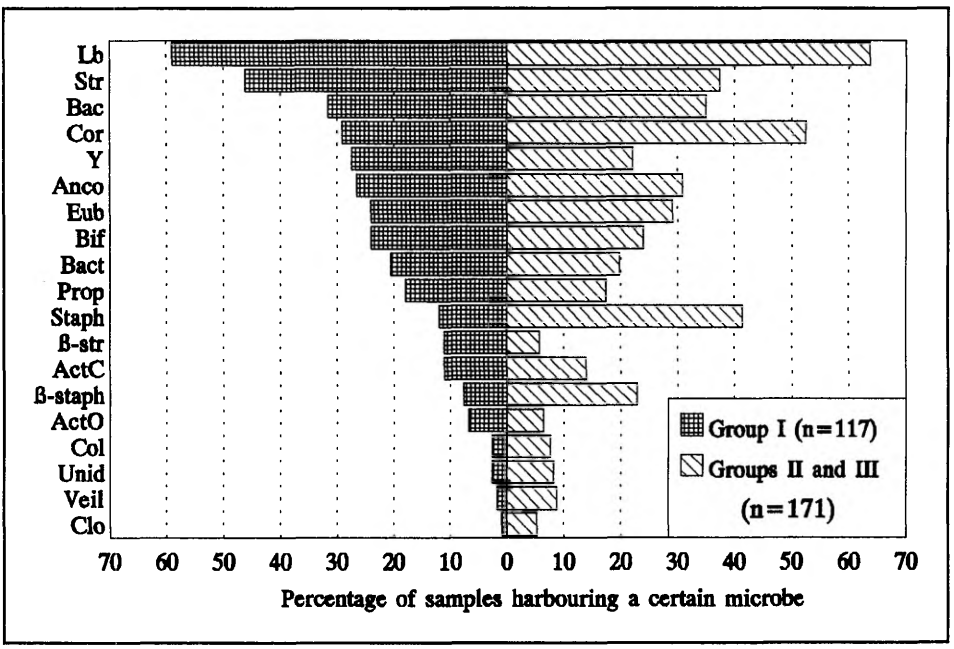


Figure 1. Microorganisms isolated from the vagina during pregnancy

subordinate microbes in each microbiocenosis. As prevailing we considered those microbes, the relative proportion of which in the total count of microorganisms exceeded 10% i.e. their number did not differ from the total count by more than one logarithm. The relative proportion of subordinate microorganisms in the total count did not exceed 10%.

It appeared that lactobacilli prevailed most frequently (Fig. 2), followed by streptococci, eubacteria, and bifidobacteria. Veillonella and coliforms never prevailed, beta haemolytic staphylococci, clostridia and coccobacteria prevailed in a few cases.

5.1.1.4. Types of the microflora

To simplify the evaluation of individually variable microflora, we applied a marker called the **type of microflora**. On the basis of estimation of stable (3–7 times during pregnancy) prevailing bacteria in the VMf, the women fell into 7 types (Table 8). Only in two cases we failed to determine the type of the VMf. Microbes of a greater pathogenic potential such as coliforms, clostridia, bacteroids, beta haemolytic streptococci, *etc.* did not form a stable part of the microflora, but occurred only in a few samples. Lactobacilli-containing types

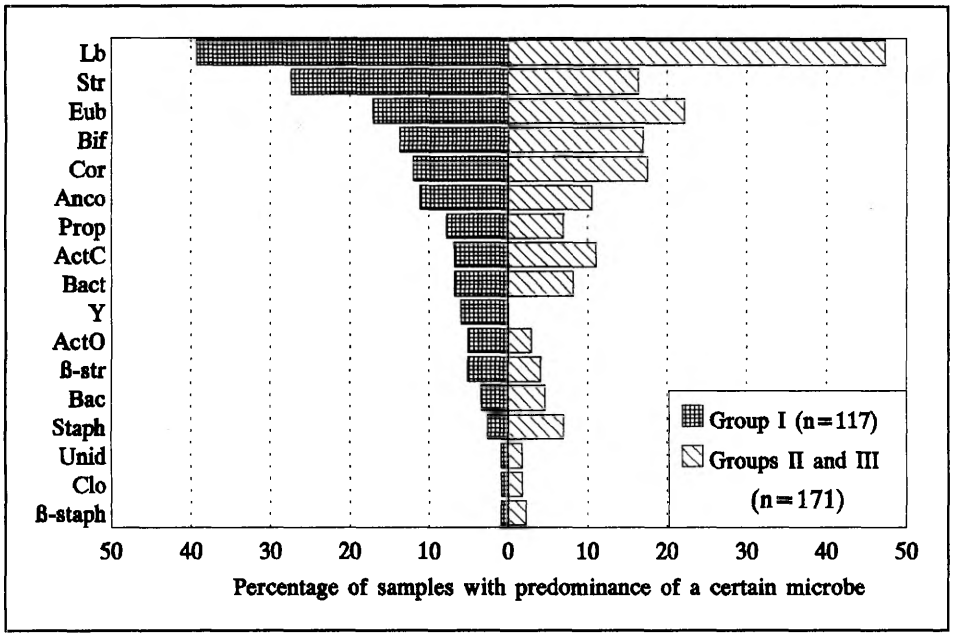


Figure 2. Frequency of predominance of different microorganisms in vaginal microflora

were the most frequent ones, although the pure lactobacilli type was found only in one woman of Group I.

5.1.2. Elaboration of methods for evaluating individual vaginal samples (Sample level)

(Paper I, II, IV)

5.1.2.1. Criteria on the basis of Gram-stained vaginal smears

To get a complete overview of the VMf, we combined the bacteriological method described above with direct microscopy of the vaginal material. In this way the state of VMf was evaluated and BV diagnosed. The criterion for bacterial vaginosis (BV) was a total score of 7 or higher; a score between 4 and 6 was considered intermediate, and a score between 0 and 3 classical.

From investigated samples, BV was diagnosed in 36.2%. Nearly half (45.7%) of all the investigations showed the intermediate VMf, the classical microflora being found only in 18.1% of the samples.

Out of 23 women, we found BV in 11. Five women had a stable BV throughout pregnancy, four women had it in most and two women barely in a

few samples. Not a single woman was found to harbour a classical microflora (score between 0 and 3) in all the testings during pregnancy.

"Clue cells" were found in 41 samples out of 42 with BV (score ≥ 7). They were present only in one case out of 53 intermediate and absent from the classical microflora, indicating a strong correlation with BV. By this we found it possible to widen the concept of "normal" vaginal flora proposed by Nugent *et al.* (1991) and to consider both classical and intermediate microflora as normal.

Table 8

Types of microflora based on the predominance pattern

Type (stable predominance pattern)	No of women			Persistent BV by Gram stain		
	Gr. I	Gr. II	Total	Gr. I	Gr. II	Total
1. Lactobacilli	1	2	3	0	0	0
2. Lactobacilli + streptococci	4	3	7	1	0	1
3. Lactobacilli + anaerobic coryneforms	2	3	5	0	0	0
4. Lactobacilli + *Eubacterium*	3	3	6	0	0	0
5. *Eubacterium*	3	2	5	0	0	0
6. Coryneforms	6	5	11	5	3	8
7. Cocci	2	1	3	2	0	2
Type indeterminable (varying microflora)	2	0	2	1	0	1

5.1.2.2. Criteria on the basis of cultivation of vaginal microorganisms

By analysing the distribution of isolated microorganisms we found some regularity in the occurrence of certain microorganisms in samples with and without BV. BV was negatively correlated with lactobacilli ($p=0.0000002$) and eubacteria ($p=0.0003$), and positively with corynebacteria ($p=0.00002$), propionibacteria ($p=0.0002$), bifidobacteria ($p=0.000006$), anaerobic cocci ($p=0.01$), beta haemolytic streptococci ($p=0.03$) and bacteroids ($p=0.01$).

To develop criteria for evaluation of the VMf, only the samples without BV were used. To get a better overview of the distribution of microorganisms, we applied the graphic expression of their relative proportions in the remaining 74 samples (Fig. 3).

For most microorganisms we could determine **their ceiling relative proportion in the microflora**, the values below which may be considered the balance and the values above the limit — the imbalance of the microflora. For streptococci the limit was 50%, for bifidobacteria 40%, for coryne- and propionibacteria 20%, for bacilli, anaerobic cocci and bacteroids 10%, for yeasts, staphylococci, beta haemolytic strepto- and staphylococci 1%.

In case of lactobacilli and eubacteria we could not determine their upper limit, their relative proportion could be up to 100%. Normally at least one of them predominated in the majority of samples; the absence of one could be considered normal if the other was present.

We could not establish criteria for the microorganisms that occurred only in a few samples and were sparsely distributed.

5.1.3. Discussion

Using two different methods — microscopical investigation of the vaginal smears and quantitative cultivation of the microbes — we examined the composition of the vaginal microflora of consecutive pregnant women.

As mentioned above, we have used some different media and methods for isolation of lactobacilli and clostridia from the women of different groups. The different MRS-media for lactobacilli did not give rise to any difference in their isolation frequency. Some difference, however, statistically negligible, was noticed in case of anaerobes as clostridia. When we began the present study, the only available method for the cultivation of anaerobic microorganisms in our laboratory was the roll-tube method. This method offers a good possibility for cultivating the anaerobes in a very stable anaerobic environment and checking the appearance of growth without preterm disturbance of the anaerobic environment, however, it is very time- and labor-consuming. This method has

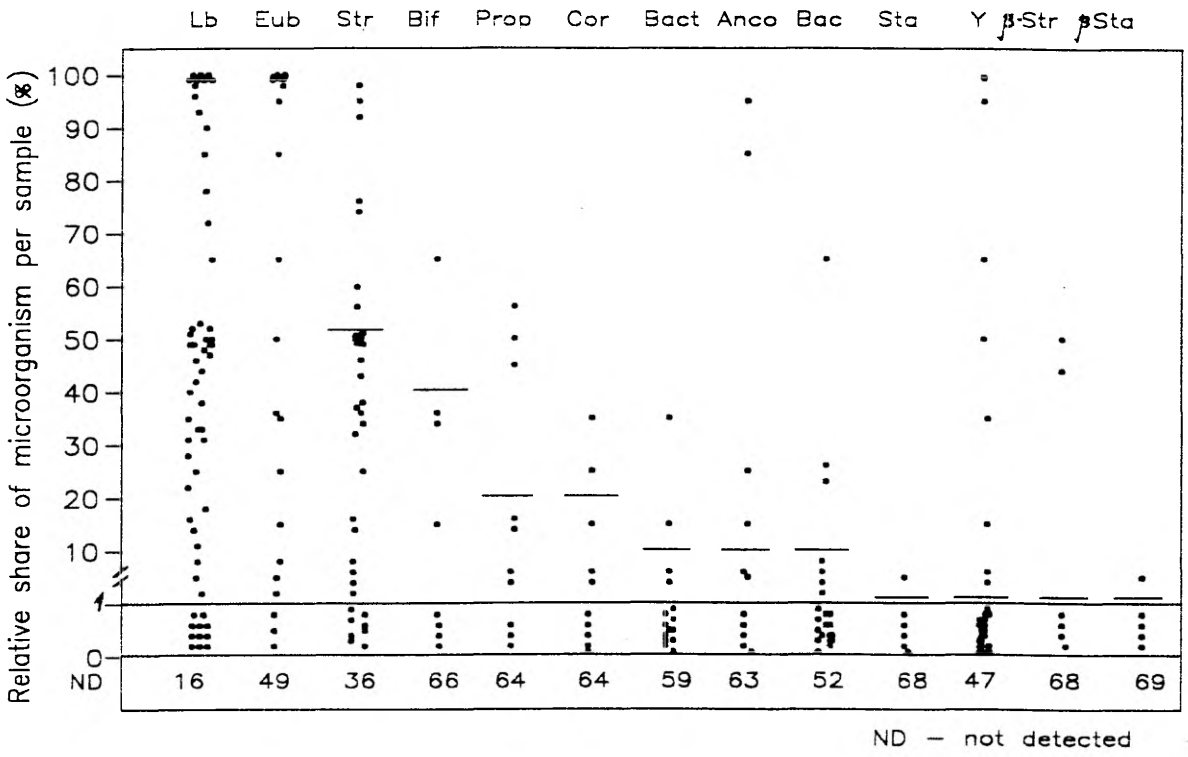


Figure 3. Ceiling proportions for particular microorganisms

been successfully used in the Department of Microbiology of University of Tartu during many years for studies of intestinal microflora (Mikelsaar and Lenzner, 1979; Mikelsaar *et al.*, 1984; Mikelsaar and Lenzner, 1988; Mikelsaar *et al.*, 1990) and for diagnostics of clinial anaerobic infections (Mikelsaar and Lenzner, 1983). Nowadays the most popular method seems to be the anaerobic glove box because of its convenience and high efficacy, however, its comparably high price keeps the other methods also viable. The fact that more than 100 different methods have been developed for cultivating the anaerobic microorganisms indicates that none of them is the best without competition.

We have compared the appearance of anaerobes colonies in roll-tubes in blood-thioglycollate-agar with the golden standard of plating microorganisms on blood-agar (Stargel *et al.*, 1978; Mitsuoka, 1980) and found several colonial characteristics well comparable with a good diagnostic value (paragraph 4.2.2.). Also the well-distinctive characteristics of microbial cells in Gram-stained preparations compared with the literature data (Holdeman *et al.*, 1977; Mitsuoka, 1980) served as the basis for grouping of anaerobes in our quantitative studies. It is noteworthy, that the aforedescribed morphological parameters as well-known characteristics of anaerobic microorganisms have still been included into the latest Clinical Microbiology Procedures Handbook (Isenberg, 1995).

We have isolated a wide variety of microorganisms in different combinations from the vagina, which corresponds to the results obtained by other investigators (Ross and Needham, 1980; Walss-Rodriguez *et al.*, 1988) and which has always made the evaluation of vaginal microflora difficult (Larsen and Galask, 1980; Galask, 1988; Redondo-Lopez *et al.*, 1990). To overcome this difficulty, we propose to determine the relative proportion of microorganisms in the microflora. Such an approach has enabled us to investigate the ecological structure of the microflora. We found a long list of microbes that in some samples could exceed the 10% limit. However, not all of these microbes could form a stable part of the microflora. Certain microbes did occur in very large numbers in a few cases only, never being stably present during the course of pregnancy. Thus, their predominance can be considered a temporary imbalance of the microflora. Our data confirm the idea of Summers and Sharp (1993) that in complicated cases (absence of the known pathogens) the quantitative cultures of vaginal microorganisms could be useful.

On the basis of the stable predominant microorganisms we could find seven types of the vaginal microflora in pregnant women. This finding is in accordance with the data of Lee *et al.* (1995) who have found sub-groups within the women harbouring a normal vaginal flora.

For most of the microorganisms we ascertained their ceiling relative proportions in the VMf as the criteria for diagnosing the imbalance of VMf. The latter were found using the natural distribution of microorganisms in the

microflora in women without BV or TAb. For lactobacilli and eubacteria we could not determine the upper limit, as their high numbers were normally present in the balanced VMf and they could substitute for one another. The reliability of our criteria was proven also by negative correlation of BV with bacteriologically determined lactobacilli and eubacteria, and positive correlation with corynebacteria, propionibacteria, bifidobacteria, anaerobic cocci, beta haemolytic streptococci and bacteroids. These relations have been found also by other investigators (Spiegel, 1991; Hillier, 1993; Larsen, 1993; Pybus and Onderdonk, 1995).

It became evident that the presence of the "clue cells" is quite a good marker of BV. Having taken into account the presence or absence of "clue cells", we considered as normal both the intermediate and the classical microflora patterns. Unfortunately, no special medium was used for *Gardnerella vaginalis*. It is difficult to distinguish this microorganism from corynebacteria only by morphology (Piot *et al.*, 1982; Catlin, 1992; Florez *et al.*, 1994) and for this reason these bacteria were grouped as corynebacteria. Also *Mobiluncus sp.* was quite a new microorganism when the present study was started, so we did not diagnose it.

* * *

Thus, we have developed methods for evaluating of the VMf of pregnant women, using both quantitative bacteriological and bacterioscopic studies and elaborating the following complex of indicators:

* on group level
  - — total count of microorganisms and occurrence of particular microorganisms;
  - — predominance pattern and types of microflora;
* on sample level
  - — ceiling relative proportion of particular microorganisms in the microflora on the basis of cultures of sample;
  - — score on the basis of direct microscopy of sample.

5.2. APPLICATION OF THE METHODS DEVELOPED FOR WOMEN WITH AN EARLY THREATENED ABORTION (Groups II and III)

Using the above complex of indicators we evaluated the state of the VMf in women with an early TAb (Groups II and III).

5.2.1. Comparison of women with and without early TAb (Paper I)

5.2.1.1. Counts of microorganisms

Various aerobic and anaerobic microorganisms were isolated from all the 117 samples of Group II women and all the 54 samples of Group III women. The amount of microorganisms throughout the course of pregnancy in Group II was 2.2-7.7 log CFU/swab, with a mean 6.7, and in the Group III 2.0–7.5 log CFU/swab, with a mean 5.5. Anaerobic bacteria prevailed in 47 (40.2%) samples of Group II, aerobic bacteria in 58 (49.6%), and the counts of aerobes and anaerobes were nearly equal in 12 (10.2%) samples. In Group III, these numbers were 16 (29.6%), 35 (64.8%) and 3 (5.6%), respectively. The number of samples with the predominance of either aerobic or anaerobic microorganisms was quite similar in all three groups ($p=0.17$).

5.2.1.2. Occurrence of microorganisms

In Group II we isolated the same different microorganisms (Fig. 1) as in Group I, in Group III we failed to find beta haemolytic streptococci. There could be found simultaneously 1–8 different microorganisms in one sample in Group II, the mean 4.7 being higher than the mean (3.6) in Group I ($p<0.05$). In Group III there were 2–8 (mean 4.3) different microorganisms in one sample, and no difference was found in comparison with Group I or II ($p>0.05$). Like in Group I, lactobacilli were the most frequent microorganisms in both Groups II and III.

Some differences were revealed in the occurrence of microorganisms: epidermic staphylococci ($p<0.01$), beta haemolytic staphylococci ($p<0.01$), clostridia ($p<0.05$), coliforms ($p=0.05$), corynebacteria ($p<0.01$), veillonella ($p=0.01$) and coccobacteria ($p<0.05$) were found more often in Groups II and III than in Group I.

5.2.1.3. Predominance pattern of the microflora

The most frequently prevailing microorganisms in Groups II and III were also lactobacilli as in Group I (Fig. 2). However, some differences were noted when analyzing the frequency of the other prevailing microorganisms. In Group I, yeasts prevailed in seven cases, in Groups II and III, never ($p=0.001$). Also, in Groups II and III, streptococci rarely prevailed ($p=0.02$).

5.2.1.4. Types of the microflora

In women of Group II we could distinguish 7 types of the vaginal microflora (Table 8), which coincided with the respective types of Group I. There were no significant differences in occurrence of these types of microflora between the two groups (I and II) of women studied ($p>0.2$). In women of Group II the lactobacilli-containing types also appeared to be the most frequent ones.

It is important to note that the stable BV revealed by Gram-stained slides was in good correlation with some types of the microflora (Table 8), such as aerobic and anaerobic coryneforms and cocci ($r=0.67$, $n=40$, $p<0.01$).

5.2.2. Evaluation of individual samples

(Paper I, II, IV)

5.2.2.1. State on the basis of Gram-stained vaginal smears

Bacterial vaginosis was found in 26.5% of the samples of Group II, the difference from Group I being statistically negligible ($p=0.19$). Two women had a stable BV throughout pregnancy, three women had it in most and four women in some samples only. Hence, in Groups I and II together seven women had a stable BV throughout the pregnancy (Fig. 4). In Group III, BV was found in 22.2% of samples in 9 (33.3%) of the women. Thus, we could not find any difference from Group I ($p=0.14$) in them.

Analyzing the data of the three groups studied (Groups I–III), we could detect BV in 29.7% of the samples. More than half (50.5%) of all the investigations of Groups I–III showed the intermediate VMf and 19.8%, the classical microflora pattern. Not a single woman was found to harbour a classical microflora (score between 0 and 3) in all the testings during pregnancy. We found a good correlation between the presence of "clue cells" and the count of different microbial morphotypes — "clue cells" were found in 80 out of 84 samples with BV (Fig. 5). At the same time, they were found only in two cases out of 143 by the intermediate and never once by the classical

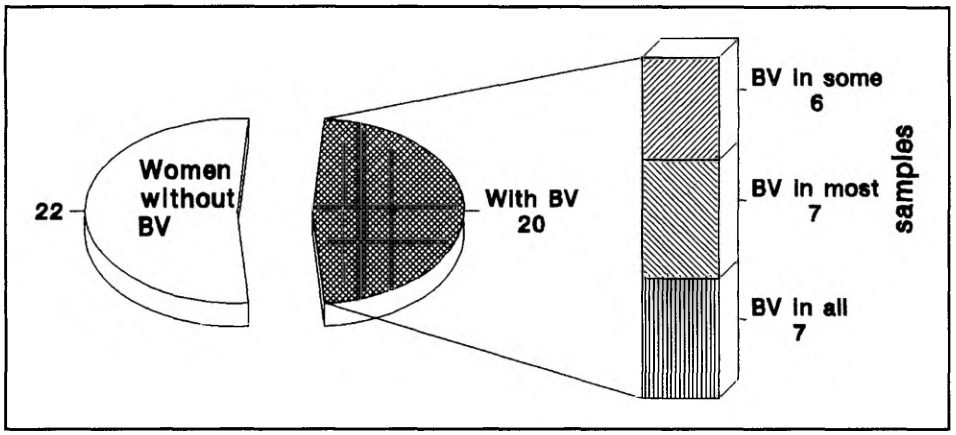


Figure 4. Occurrence of BV among pregnant women of Groups I and II

microflora ($r=0.949$, $n=283$, $p<0.00001$).

When we compared the first investigation of microflora in women of Groups II and III at the moment of threatened abortion with the first investigation pregnant women without threatened abortion (Group I), we could not find any difference in the presence of BV ($p=1.0$).

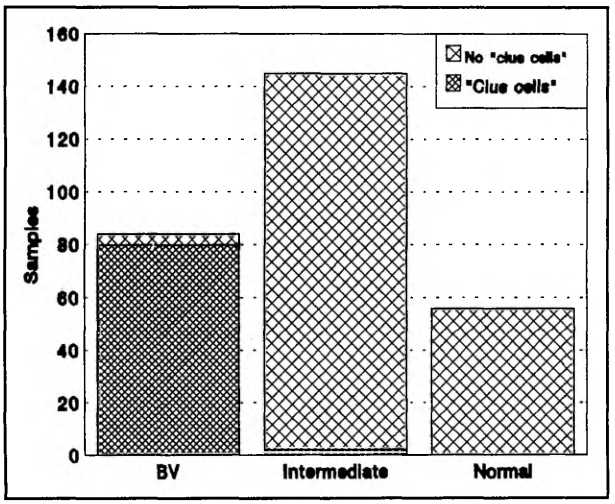


Figure 5. Occurrence of "clue cells" in different vaginal microfloras

5.2.2.2. State on the basis of cultivation of vaginal microorganisms

By using the proposed ceiling relative proportions of microorganisms in the VMf (paragraph 5.1.2.2.) we evaluated the individual samples of women of Groups II and III. We found that the changes in individual microfloras could involve 1 to 4 microorganisms simultaneously.

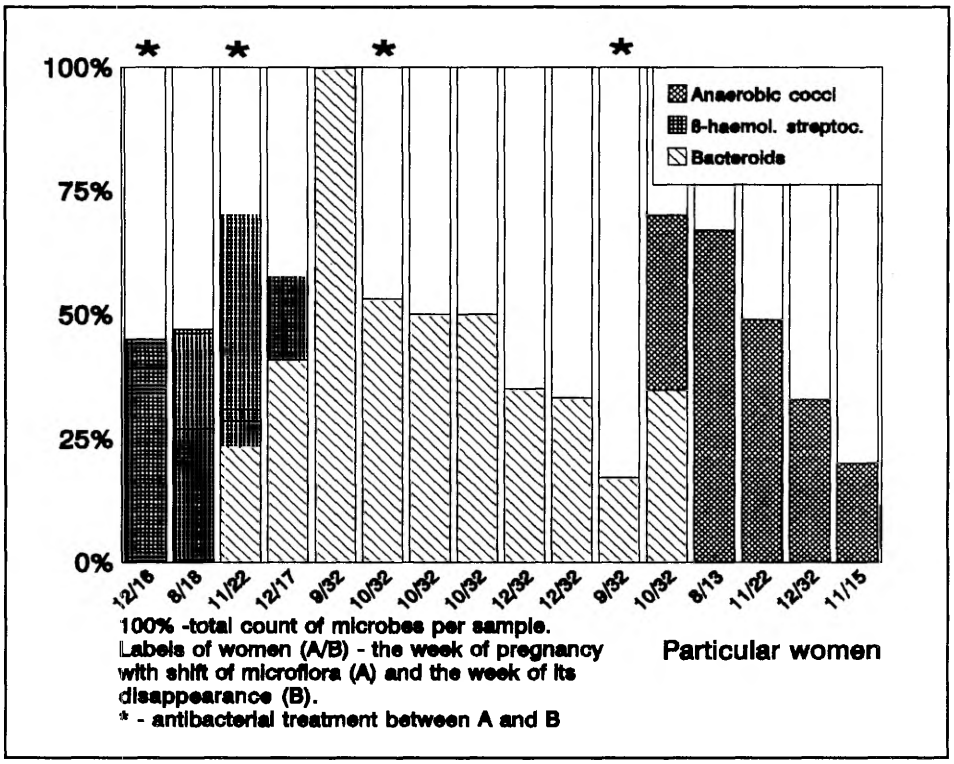


Figure 6. Pregnant women with predominance of some opportunistic pathogens at the time of TAb

At the time of a TAb (in the first examination), we revealed some significant shifts in the relative proportion of some opportunistic microorganisms in 16 (34%) women of Groups II and III (Fig. 6). For instance, in four patients the relative proportion of beta haemolytic streptococci had increased in comparison with their balance criterion $<1\%$ (17%, 45%, 47% and 51%, respectively), in ten patients the proportion of bacteroids was higher (26%, 41%, 33%, 99%, 17%, 63%, 35%, 50%, 50%, 35%; criterion $<10\%$) and in five women that of anaerobic cocci (20%, 49%, 67%, 33%, 35%; criterion $<10\%$) had increased.

We found that bacterioscopically diagnosed BV coincided with bacteriologically revealed changes in the microflora in 98% of the cases, which confirmed the reliability of our criteria.

5.2.3. Association of changes in VMf with adverse pregnancy outcome

Adverse pregnancy outcome appeared to be highly frequent in Groups I–III, no difference was found between the women with and without TAb ($p=0.74$).

We could detect no correlation either between BV in the last sample before delivery and an adverse pregnancy outcome ($r=-0.027$, $n=42$, $p=0.87$), or between a stable BV and adverse pregnancy outcome ($r=-0.135$, $n=42$, $p=0.39$) in Groups I and II.

When we evaluated the microflora composition of the last sample before delivery in women with and without adverse pregnancy outcome by the criteria proposed by us (see paragraph 5.1.2.2.), we found changes in microflora composition in five cases with and ten cases without adverse pregnancy outcome, the difference being statistically nonsignificant ($p=0.22$). The changes included staphylococci in two women, and corynebacteria, bacteroids and bacilli, each in one woman with adverse pregnancy outcome.

The women of Groups II and III whose first samples had revealed significant shifts in the relative proportion of some opportunistic microorganisms which later disappeared (Fig. 6) did not have adverse pregnancy outcome more frequently than the other women ($p=0.28$).

5.2.4. Discussion

Applying classical characteristics of microflora investigations (frequency of occurrence of particular microorganisms, counts of microbes *etc.*) to comparison of VMf of pregnant women with and without TAb we have revealed some shifts in the quantitative composition of their microflora, however, mainly nonspecific and not easily understandable. Our new approach enables us to look on the relative proportion of opportunistic microorganisms in the complex microflora as a whole and to compare it with the data of bacterioscopic investigation. The results of quantitative bacteriology were in good accordance with the data of examination of direct smears of vaginal samples. We have shown that in case of TAb in some one third of the women, the imbalance of vaginal microflora was found related to different opportunistic bacteria as ß-haemolytic streptococci, bacteroids and anaerobic cocci. Also Daugaard *et al.* (1988) has shown an association between the occurrence of GBS in the genital tract and spontaneous abortions. However, this comparatively low percentage of women with disturbed microflora seems to confirm the suggestion that in most cases the shifts in VMf are not the etiological factors of TAb but, more likely, the changed microflora may reflect the hormonal dysbalance of the

organism, since we found that later during hormonal therapy these particular changes disappeared.

We could detect BV quite frequently (in 31.3% of all the samples) in the pregnant women examined. Its incidence nearly corresponds to the data provided by other authors who have found BV in 10–26% of pregnant women (Cristiano *et al.*, 1989; Thomason *et al.*, 1991; Kurki *et al.*, 1992; Platz-Christensen *et al.*, 1993). It is interesting to note that in some women BV was unstable, whereas others had it throughout their pregnancy. Our study confirmed the suggestion of Thomasen *et al.* (1992) that gynaecologists can rely on "clue cells" when diagnosing BV hastily without counting microbial morphotypes. Two types of the VMf revealed by bacteriological studies (predominance of coryneforms and cocci) appeared to be pathological because of their strong correlation with bacterioscopically diagnosed BV.

The changes in the VMf in an early TAb later disappeared and did not affect the pregnancy outcome. Nor could we find any correlation between the changes in vaginal microflora just before delivery and adverse pregnancy outcome. Also Carroll *et al.*, (1996) have found that cultures of lower genital tract provide poor prediction of intrauterine infection in PROM, yet some other authors have described the relation between VMf and adverse pregnancy outcome (Kurki *et al.*, 1992; Holst *et al.*, 1994). The adverse pregnancy outcome might be associated with the altered cervical microflora or many other different factors, and in case of the women we studied it could more often than not have been connected with other factors, such as emotional stress for example (Syrica, 1990), since many of the women involved were students.

5.3. DYNAMICS OF THE VAGINAL MICROFLORA DURING PREGNANCY

We studied the composition of VMf of the women of Groups I and II repeatedly to find out what changes take place during pregnancy. We also investigated the possible influence of antibiotics on the lactoflora of pregnant women (Groups V and VI).

5.3.1. Stability of the VMf during pregnancy (Groups I AND II)

(Paper I)

We observed every woman's predominance pattern of microflora in all testings during pregnancy. The prevailing microbes could be the same in all (100%) the samples of a certain woman, but on the average in 62.3% in Group I and in

62.1% in Group II. Even after antimicrobial treatment (Table 4) the predominance pattern of microflora remained unchanged in all women for the next investigation performed 2–7 weeks after the completion of the therapy. Thus, the microflora of any one particular individual appeared to be quite stable during pregnancy.

5.3.2. Dynamical changes in the VMf during pregnancy

(Paper I, II, IV)

5.3.2.1. General tendencies (Groups I and II)

In total counts of microbes per sample during different periods of pregnancy several nonsignificant fluctuations (below one logarithm), very similar in both Groups I and II, could be noted. Some well expressed dynamical changes could be noticed about particular microorganisms. The relative share of lactobacilli in the total count of microbes increased in Group II until the penultimate investigation (between the 32nd and 34th week), but immediately before birthgiving some decrease was observed (Fig. 7). In Group I, the same tendency was observed, but, the maximum occurred somewhat earlier, between the 24th and 26th week of gestation. The increase was significant in both groups ($r=0.69$ and $r=0.80$, respectively).

The relative proportion of staphylococci increased during pregnancy in both groups ($r=0.62$ and $r=0.63$, respectively), whereas the counts of propionibacteria ($r=-0.82$ and $r=-0.66$) and anaerobic cocci ($r=-0.56$ and $r=-0.85$) decreased.

In Group II, a decrease was observed in the counts of beta

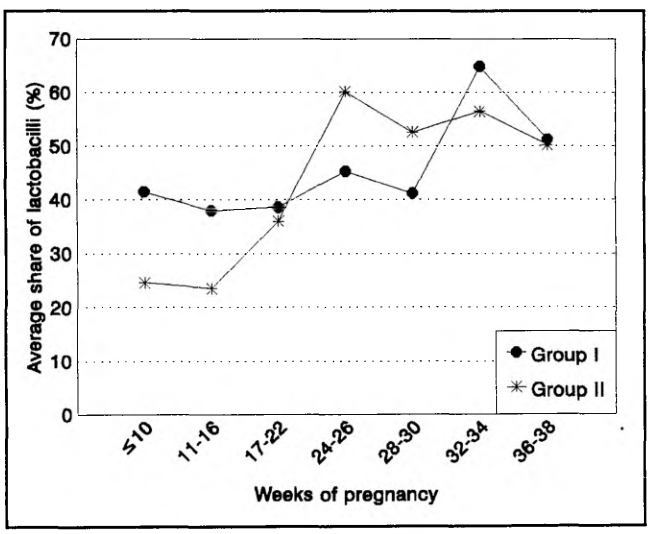


Figure 7. Average relative proportions of lactobacilli in vaginal microflora in women harbouring them

haemolytic staphylococci (r=–0.70), beta haemolytic streptococci (r=–0.89), bacteroids (r=–0.51) and bifidobacteria (r=–0.88).

The incidence of BV detected on the basis of Gram stained smears decreased during pregnancy (r=–0.62; Fig 8). Also the mean score of the VMf decreased during pregnancy (from 5.2 to 4.7).

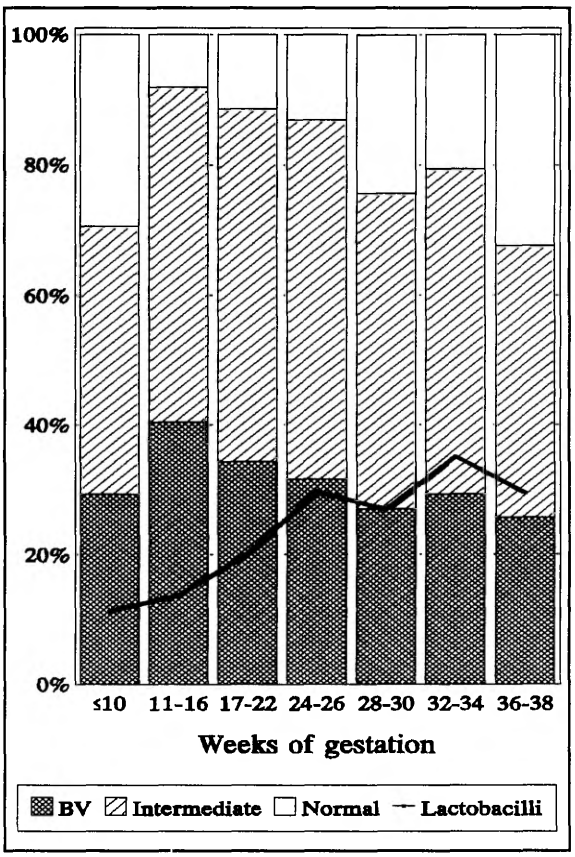


Figure 8. Dynamics of the relative proportion of lactobacilli and occurrence of BV during pregnancy in Groups I and II

5.3.2.2. Observations on changes of the VMf during pregnancy in individual women (Groups I, II and III)

We observed the course of pregnancy of certain women using the microflora balance criteria elaborated by us. This approach enabled us to find out whether the imbalance of microflora could be temporary or persistent, and if it was associated with the same or changing micro-organisms in one woman. We found 20 women of Group I displaying some changes in 1–6 samples (median 2), and 17 women of Group II with changes in 1–7 samples (median 3). In most individual women the imbalance was caused by alternating microorganisms.

By observing the women with TAb (Groups II and III) whose first samples had displayed significant shifts in the relative proportion of some opportunistic microorganisms (described in paragraph 2.2.2.), we found that in all cases these particular changes disappeared later (Fig. 6). In 4 cases antibacterial treatment for several reasons was used between these two investigations.

5.3.3. Influence of antibiotics on vaginal lactoflora (Groups V and VI)

(Paper VI)

We studied the susceptibility to 15 antibiotics of vaginal lactobacilli isolated from pregnant women in Estonia and USA. All the 36 strains of lactobacilli under investigation were susceptible to ampicillin, amoxicillin and imipenem (Table 9). A few strains were resistant to penicillin, cefotaxime, erythromycin and doxycycline. Some 13–17% of the strains were resistant to cefazoline, ceftazidime and vancomycin, nearly a quarter to clindamycin, about half of the strains were resistant to gentamicin and cefoxitin. We could observe high numbers of strains resistant to ofloxacin and aztreonam among lactobacilli investigated by us. Vancomycin-resistant strains of the lactobacilli tended to be ofloxacin-susceptible, and *vice versa* ($r=-0.81$, $p<0.05$).

The lactobacilli of Estonian (Group V) and USA women (Group VI) showed different susceptibility patterns in case of ceftazidime (5.3% of resistant strains in Group A and 29.4% in Group B, $p=0.05$) and cefoxitin (87.5% and 23.5%, respectively, $p=0.0003$). In case of cefazoline (5.3% and 23.5%) and vancomycin (5.3% and 23.5%) the difference was statistically not significant (Fig. 9).

Table 9

Susceptibility of vaginal lactobacilli (n=36) to antibiotics

Group of antibiotics	Antibiotics	Percentage of isolated strains of lactobacilli (n=36)	
		susceptible	resistant
Penicillins	Penicillin	97.2	2.8
	Ampicillin	100	0
	Amoxicillin-clavulanate	100	0
Cephalosporins	Cefazolin	86.1	13.9
	Cefotaxime	97.2	2.8
	Ceftazidime	83.3	16.7
	Cefoxitin	45.5	54.5
Other beta-lactams	Aztreonam	8.3	91.7
	Imipenem	100	0

Group of antibiotics	Antibiotics	Percentage of isolated strains of lactobacilli (n=36)	
		susceptible	resistant
Aminoglycosides	Gentamicin	60.6	39.4
Quinolones	Ofloxacin	20.6	79.4
Tetracyclines	Doxycycline	97.0	3.0
Macrolides	Erythromycin	97.2	2.8
Lincosamides	Clindamycin	72.7	27.3
Glycopeptides	Vancomycin	86.1	13.9

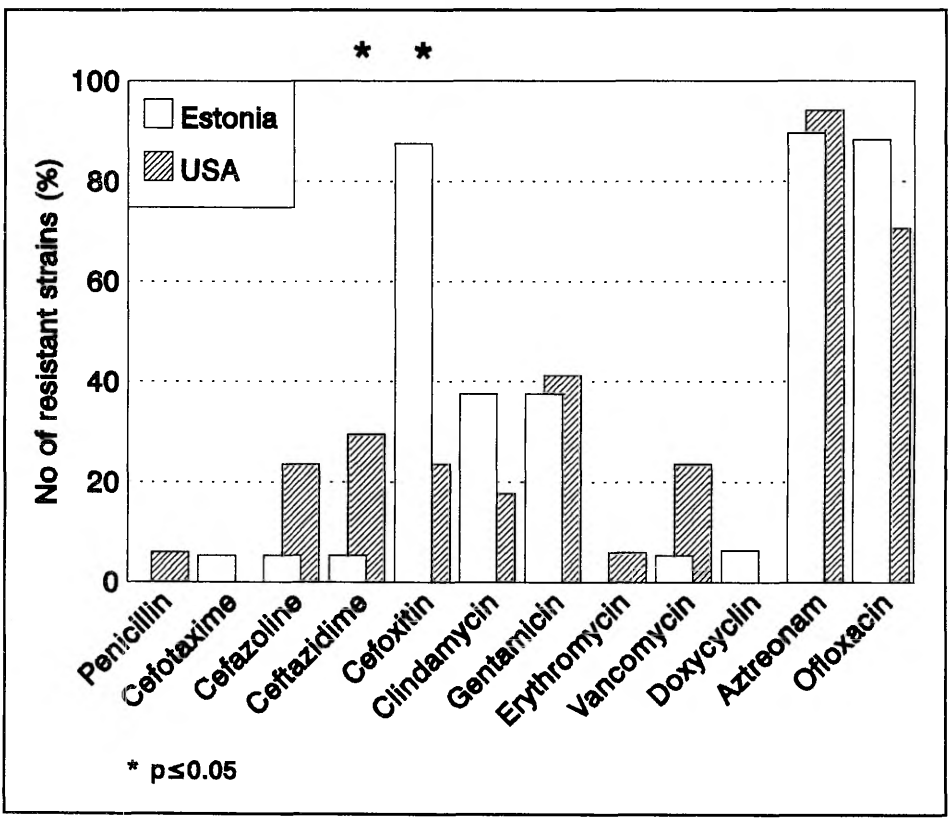


Figure 9. Number of resistant strains among vaginal lactobacilli isolated from Estonian and USA women

5.3.4. Discussion

The VMf has a tendency to maintain its stability, which could be shown by the persistent predominance patterns (types) of the microflora. It is especially interesting that even antimicrobial treatment did not change the individual type of microflora, as the stable predominance pattern was restored within 2–7 weeks after treatment. This finding is in good correlation with the data of previous studies (Grossman and Adams, 1979; Agnew and Hillier, 1995). Also Larsen (1993) has stressed that the stability of the VMf emphasizes the point that the microorganisms that colonize this tissue are well adapted to their environment and even when perturbation of the ecosystem occurs, recolonization is likely. This stability is apparently associated with individual receptor pattern of the host's cells (Mikelsaar *et al.*, 1989). Some unstable fluctuations in the microflora composition may be related with the circumstance that the vaginal microflora is a very open system and is periodically in contact with the outward microflora. Stability of microflora is also characteristic of gastrointestinal microflora, which has been found to be stable even as long as during thirteen years (Mikelsaar, 1992).

The total incidence of BV decreased during pregnancy, which has been found also by Platz-Christensen *et al.* (1993). This confirms the activity of regulatory mechanisms aimed at stabilizing the VMf for delivery. The tendency of VMf to become more lactic-acid producing as gestation advances, seems to be associated with the hormonal changes during pregnancy. This may directly influence the microorganisms, since various concentrations of oestrogens and progesterone change the adhesion capacity of microbes to the cells of a macroorganism (Kalo and Segal, 1988; Brilene *et al.*, 1989; Brilene, 1990). These changes appeared earlier in Group II, evidently due to hormonal treatment with *Turinal*. It can be supposed that these changes serve as a preparation for delivery when the newborn gets contaminated with its first microbes (Davies and Gothefors, 1984; Deschekina *et al.*, 1990; Redondo-Lopez *et al.*, 1990). It is more difficult to predict the behavior of the other microorganisms during pregnancy since their correlations with time are weaker, however, the decreasing tendency of some anaerobic bacteria could be noted in both groups studied.

We could observe that vaginal lactobacilli are not uniform as to their susceptibility to antibiotics. Also Hamilton *et al.* (1994) have found that lactobacilli are heterogenous in terms of tolerance to antibiotics and so it is difficult to predict their sensitivity patterns. Our results correspond to data of a number of earlier studies showing that most strains (up to 80%) of lactobacilli are susceptible to such betalactams as penicillins and imipenem (Bantar *et al.*,

1991; Koneman *et al.*, 1992; Hamilton *et al.*, 1994), but also to erythromycin, clindamycin and doxycycline (Yao and Moellering, 1995). The curious susceptibility of lactobacilli to cephalosporins might be explained by additional mechanisms of action like triggering of membrane-associated autolytic enzymes or inhibition of bacterial endopeptidase and glycosidase (Yao and Moellering, 1995). The strains of lactobacilli in Estonian women appeared to be more susceptible to ceftazidime, but more resistant to cefoxitin. This seems to suggest that the longer experience in use of both in USA and the undiscriminating use of antimicrobial drugs allegedly characteristic of postsocialist Estonia do not seem to be the reasons for different susceptibility patterns of vaginal lactobacilli in both countries. It is more likely that this is associated with the individual differences in human microflora species composition (Mikelsaar, 1992). It should be important for clinicians to know that treatment with aztreonam and ofloxacin seems to be safer for vaginal lactoflora in both countries since they have been found to be nonteratogenic (Shepard, 1992; Friedman and Polifka, 1994).

* * *

All these findings confirm the usefulness of observing the VMf dynamically during pregnancy for scientific purposes, since pregnancy makes this survey like a model of microbial ecology of the vagina. Several unsolved problems — how the regulation mechanisms of the VMf actually work, why the microflora balance is sometimes distorted, *etc.* — could be solved by surveying pregnant women.

Our suggestion to clinicians is that the vaginal microflora should at first be evaluated by microscopic examination and simple tests (pH, KOH-test), and only in unclear complicated cases (the absence of STD, candidiasis and bacterial vaginosis) by estimation of the quantitative composition of microflora to reveal the predominance patterns of microorganisms.

The above methods for the evaluation of the VMf have been discussed and approved at some international conferences (SOMED meetings — 1992 in Helsinki, 1993 in Boston) and are employed in the laboratory of microbiology at the Maarjamõisa Hospital of University of Tartu.

5.4. ROLE OF THE MOTHER'S MICROFLORA IN THE INITIAL MICROBIAL CONTAMINATION OF THE NEWBORNS

(Paper III, V)

To study the influence of mothers' vaginal microflora on the first microbial contamination of the newborn, the mother-baby pairs were investigated immediately after delivery and during the first week of stay in maternity hospital. Also we looked for some opportunistic pathogens in pregnant womens' delivery tract as one possible source for colonizing the baby.

5.4.1. Vaginal microflora during delivery (Group A)

We succeeded in investigating the 19 above-mentioned women of Groups I and II immediately after vaginal delivery. The total count of microbes per swab during delivery appeared to be smaller than at the end of pregnancy. A few days after delivery (confirmed in 12 women) their count returned to the former level (Fig. 10).

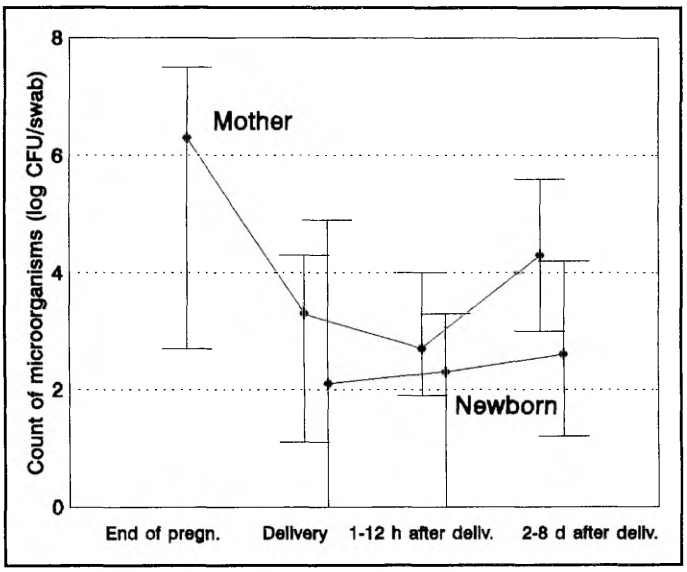


Figure 10. The variety and medians of the total counts of mothers' vaginal and newborns' ear canal microorganisms before, during and after delivery

Occurrence of the microbes isolated from the vagina during delivery generally coincided with that of the microbes isolated during the last weeks of pregnancy. However, occurrence of lactobacilli and yeasts decreased, whereas that of streptococci increased. During delivery, the prevailing and subordinate microbes were estimated only in cases in which the

total count of microbes was > 100 CFU/swab to avoid registration of random distribution of microorganisms in scarcely colonized areas. We found that 13 groups of microbes could prevail (Fig. 11), streptococcal pre-dominance being most frequently assessed.

5.4.2. Comparison of the mothers' vaginal and the newborns' ear skin microflora during delivery (Group A)

The distribution of the counts of microorganisms in the newborns' external ear canal was quite similar to their mothers' vaginal one: most of them — 84% of mothers and 74% of newborns — harboured high numbers of microorganisms (> 100 CFU/swab).

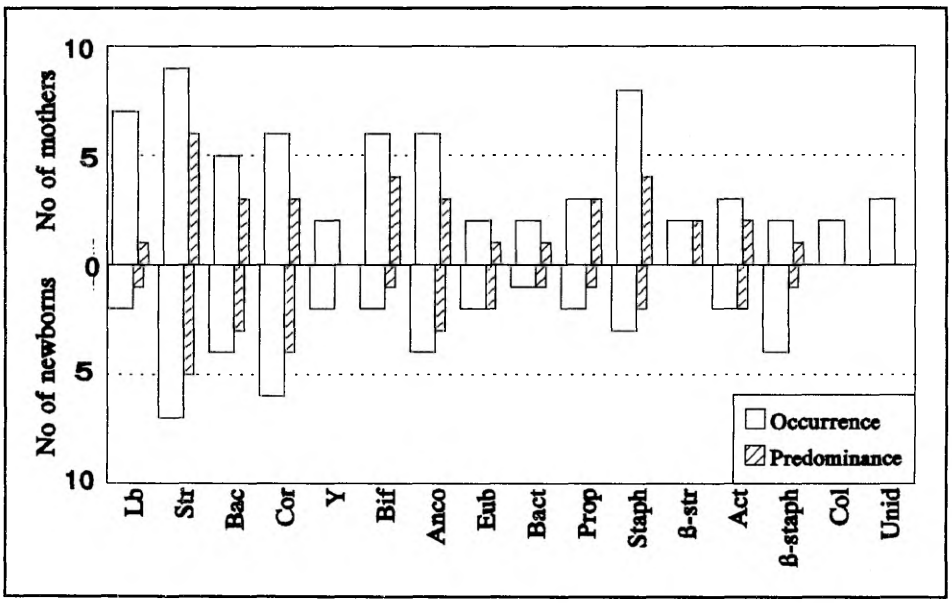


Figure 11. Occurrence and predominance of microorganisms in vaginal samples of mothers and ear samples of newborns (Group A)

Streptococci were the most frequently occurring microorganisms in the mothers' vagina and newborns' ear skin (Fig. 11). Various individually different combinations of up to 8 microbes were observed per sample in mothers and up to 6 microbes per sample in babies.

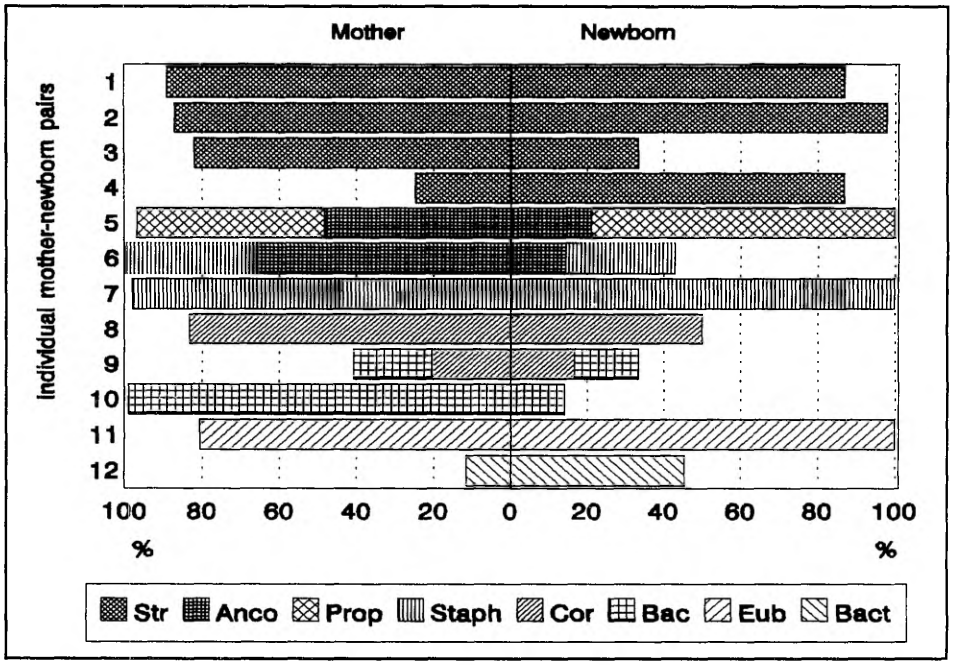
As in mothers, the prevailing microbes were estimated only in heavily contaminated newborns (>100 CFU/swab). Comparing the predominance patterns of the mothers' vaginal and the children's ear skin microflora, we found streptococci to be the most frequent prevailing microbes: in 6 mothers and 5 newborns. As regards opportunistic microorganisms, we observed that yeasts never predominated either in mothers or in their newborns. Beta-haemolytic staphylococci occurred among the predominant microbes only in unpaired samples (one mother and one newborn), thus comprising less than 25% of the total count of microbes. Beta-haemolytic streptococci predominated only in one mother (20% of the total count) and never in newborns.

We found one or two similar predominant microorganisms in 12 mother-newborn pairs (85%) from among these 14 pairs in which the newborn was heavily contaminated (Fig. 12). Streptococci were the most frequent common predominant microorganisms, occurring in 4 mother-newborn pairs. In the remaining pairs, we found different similar predominant microorganisms: staphylococci, bacilli, corynebacteria and anaerobic cocci each in 2 pairs; bacteroids, propionibacteria and eubacteria each in one pair. In addition to that, up to 4 similar subordinate microorganisms could be detected in the investigated pairs.

5.4.3. Comparison of the mothers' vaginal and the newborns' ear skin microflora after delivery (Groups B and C)

The spectra of microorganisms of all the mother-baby pairs in Group B (4 pairs, examined between 1 and 12 hours after delivery) were different. No microbes were found in one child. Similar prevailing microbes were found in one pair, similar subordinate ones — in one other pair. Coliforms were present in one mother, but we could not find them in her baby. This small group seemed to be quite similar to Group A.

In Group C (11 pairs, examined between 2 and 8 days after delivery) the mothers most frequently harboured coliforms (9 out of 11 mothers), followed by streptococci and bacilli (Fig. 13). The newborns harboured most frequently beta haemolytic staphylococci (9 out of 11), coryne- and propionibacteria. The most frequent prevailing microbes were beta haemolytic streptococci in mothers and beta haemolytic staphylococci in newborns. Similar prevailing microbes were found in 7 pairs (1–3 similar microbes per each pair).



100% — total count of microorganisms per sample

Figure 12. Similar predominant microorganisms in twelve mother-baby pairs of Group A

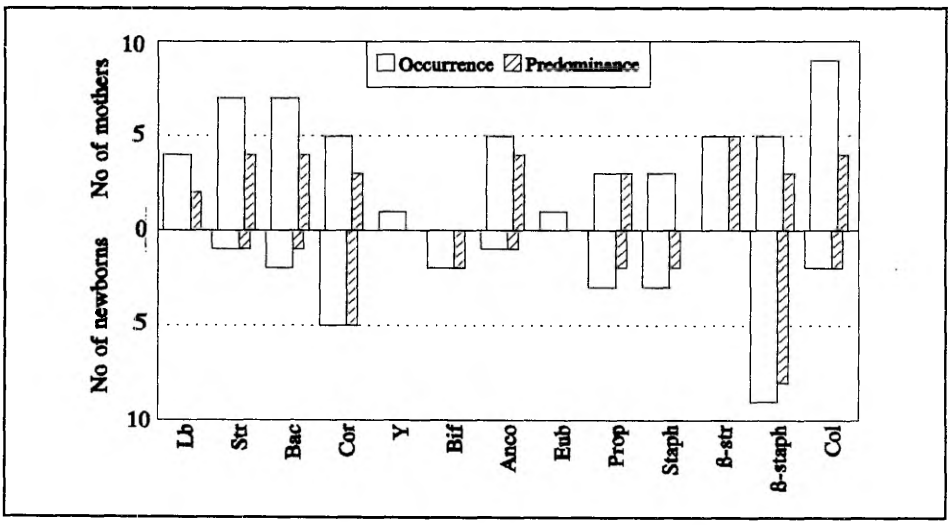


Figure 13. Occurrence and predominance of microorganisms in vaginal samples of mothers and ear samples of newborns (Group C)

5.4.4. Prevalence of *Clostridium difficile* and group B streptococci in the delivery tract of pregnant women and their putative transfer to newborns (Groups IV and V)

To study the possible source of the initial contamination of the newborn with *C. difficile* and GBS we studied the presence of these microorganisms in pregnant women.

We isolated **clostridia** from the vagina of one woman out of 23 (4.3%) in Group I, in three women out of 19 (15.8%) in Group II and in three women out of 27 (11.1%) in Group III. None of their investigated newborns harboured *Clostridium sp.* in ear skin microflora. We did not determine the species of isolated clostridia in these groups of women.

To establish the presence of *C. difficile* in delivery tract at the end of pregnancy, we investigated 22 women (Group IV) in the third trimester of pregnancy using selective media. We isolated clostridia from the vagina in 7 women out of 22 (32%) and none of them had *C. difficile*. The relative proportion of clostridia was comparatively low — 1% of the total count as median value. In 6 cases out of 7 the counts of lactobacilli were higher or equal to clostridia.

From rectal samples of these women clostridia were isolated in 6 cases (27%), one woman harboured *C. difficile*. The relative proportion of clostridia in faecal samples was also low — median 1% of the total count. Only in one case *Clostridium sp.* was found in vagina and rectum simultaneously.

We isolated **ß-haemolytic streptococci** from the vagina of ten woman out of 23 (43.5%) in Group I, in eight women out of 19 (42.1%) in Group II and in none women of Group III. None of their newborns investigated by us harboured ß-haemolytic streptococci in ear skin microflora. We did not determine the species of isolated streptococci in these groups of women.

To find out the occurrence rate of GBS among pregnant Estonian women we studied 30 consecutive pregnant women (Group V). We found ß-haemolytic streptococci in eight women, non of them was GBS.

5.4.5. Discussion

This study demonstrated that, at birth, there was a great similarity both qualitative and quantitative between the individual microflora of mother's vagina and her newborn's ear. The degree of colonization of the newborn

significantly correlated with the count of microorganisms in its mother's vagina. In most of the investigated individual mother-newborn pairs we could reveal similar prevailing microorganisms. There were no cases of the mothers and their newborns harbouring similar prevailing potentially pathogenic microorganisms.

Pertaining reports offer some conflicting results. Thus, the investigation by Graham (1975) has shown that only 25% of healthy neonates harbour microorganisms on their skin immediately after birth and the latter are the same as in the vagina. Ivanov and Kulish (1989) claimed that the contamination of healthy neonates with microbes varied in different clinics in the range between 7% and 30%, the most frequent contaminants being epidermic staphylococci. On the other hand, Sycheva *et al.* (1986) found microorganisms on the conjunctiva of 83% of neonates and the microbes resembled those found on mothers' skin. It seems that the results may depend on the one hand on the area of the baby's body studied, and on the other hand, on the methods of sampling and cultivation of microorganisms.

The individuality of mother's VMf revealed during pregnancy was reflected also in their newborns. It has been demonstrated also in previous studies while screening risk-of-infection newborns due to the contamination with opportunistic pathogens (Ross and Needham, 1980; Lee *et al.*, 1989; Scheven and Ziegler, 1990; Torres-Alipi *et al.*, 1990). However, all the numerous investigations have in most cases only registered either the presence or absence of potential pathogens, and their value in predicting the infectious agents is no doubt limited (Germain *et al.*, 1994). To overcome this drawback, we applied our method of determining the predominance patterns.

The balance of normal microflora is thought to be responsible for the colonization resistance, the presence of beneficial microorganisms avoids the colonization by opportunistic ones (Redondo-Lopez *et al.*, 1990; Onderdonk and Wissemann, 1993). Hence, the colonization of the newborn by normal microflora during delivery is important for its health (Mikelsaar *et al.*, 1989). Despite this importance, we have not found any data about microecological relations existing between the beneficial and opportunistic microorganisms in healthy newborns at the moment of delivery as compared with their mothers' VMf, however, one semiquantitative study comparing the mother's perineal and her newborn's microflora, has been performed (Mikelsaar *et al.*, 1989). This study confirmed our data about the great interindividual diversity of both vaginal and the newborn's microfloras and their similarity in individual pairs.

We could find lactobacilli in mothers more frequently at the end of pregnancy than during delivery and their relative proportion in microbiocenosis also decreased in this period. Also Ahtonen (1994) has found lactobacilli during delivery in the vagina only in 38–52% of women. The total count of microbes per swab during delivery in our study was also smaller than at the end of

pregnancy. Some days after delivery their count returned to the former level. Only in two cases were maternal lactobacilli transferred to newborn, the most frequent microorganisms in babies immediately after birth being staphylo- and streptococci. A possible reason for these changes may be the repeated treatment of mothers' genital tract with disinfectants in the maternity hospital (Juliano *et al.*, 1992). Mikelsaar *et al.* (1989) who have studied perineal microflora immediately at admission to maternity hospital, before treatment with disinfectants, have found a greater incidence of lactobacilli (85%). On the other hand, we found that this treatment did not remove beta haemolytic streptococci and coliforms. Moreover, it can be supposed that frequent occurrence of coliforms, beta haemolytic streptococci and beta haemolytic staphylococci in the mothers and newborns studied between 2 and 8 d after delivery may be due to the destruction of the normal VMf and therefore result in a decreased acquisition of beneficial microorganisms by the newborn. These results are in good accordance of Rotimi and Duerden (1981) who have found streptococci to be the commonest microbes in the mouth of the newborn just after birth and *S. aureus* in 87% of the babies on the sixth day after birth. Adverse colonization of newborn may be caused, on the one hand, by selective attraction of neonates for adherence of streptococci, as it was described by Long and Swenson (1977) in oral mucosa of newborns. On the other hand, the results may depend on the relatively higher resistance of cocci to disinfectants as compared with lactobacilli (Juliano *et al.*, 1992).

Consequently, the extensive vulvar cleansing during labour reduces the number of microorganisms, but also leads to a selective transfer of maternal bacteria, unfortunately excluding lactobacilli. Evidently, the idea of decontamination of the birth canal by nowadays antiseptics (chlorhexidine) before delivery (Burman *et al.*, 1992; Henrichsen *et al.*, 1994) does not work properly. Several authors have presumed that modern obstetric practices during birthgiving (incl. extensive cleansing of the perineum with disinfectants) are associated with impaired colonization of the neonate (Lundequist *et al.*, 1985; Jörbeck *et al.*, 1990; Hanson *et al.*, 1990; Hall *et al.*, 1990; Ahtonen, 1994) and it has been demonstrated that lack of lactobacilli and bacteroids correlates with the development of necrotizing enterocolitis in *C. perfringens*-colonized infants (Ahtonen, 1994). Some recent studies have shown that chlorhexidine only modestly reduces group B streptococcal vertical transmission (Adriaanse *et al.*, 1995).

We did not find in the pregnant women of Southern Estonia studied by us any extensive colonization of delivery tract either by *C. difficile* or GBS, however, by personal communication (Dr. Krista Lõivukene) GBS has been isolated from the vagina in 6% of Estonian women suffering from several gynaecological disorders. The aforementioned microorganisms have been frequently found in newborns hospitalised in neonatal intensive care units in

Finland and Sweden (Knoop *et al.*, 1993; Ahtonen, 1994), but, similarly to our findings, the investigators did not find these microorganisms in neonatal wards. The reason may lie in the microecological relations we have discovered between the potentially predominant microorganisms of vaginal and subsequently the newborns' skin microflora (lactobacilli, eubacteria, streptococci), which control the amount of these opportunistic bacteria. However, due to intensive antibacterial treatment these favourable relations may get disturbed. According to this point of view it is extremely important in treatment of pregnant women for different infections, e.g. urinary tract infections, to choose lactobacilli-saving antibiotics like aztreonam and ofloxacin assessed by us.

We conclude from our study that the predominance pattern of the mother's genital microflora has a significant influence on the initial microecological relations of her newborn.

* * *

Some practical recommendations:

1) to diagnose bacterial vaginosis in pregnant woman repeatedly before prescription of treatment;
2) to consider the occurrence of "clue cells" satisfactory for rapid diagnosing of bacterial vaginosis by clinician;
3) to use quantitative bacteriological analysis for the diagnosing of imbalance of vaginal microflora in complicated cases (absence of STD, candidiasis or bacterial vaginosis);
4) to use, if possible, for antibacterial treatment aztreonam or ofloxacin as antibiotics harmless to vaginal lactoflora;
5) in cleansing the woman's genital tract in childbirth the strong disinfectants should be replaced by ordinary washing devices.

6. CONCLUSIONS

1. A special methodology for evaluating the vaginal microbial ecosystem of pregnant women has been developed, applying both quantitative bacteriological and bacterioscopic methods of investigations. The predominance pattern, the types of vaginal microflora and the ceiling relative proportions were established as novel characteristics. The ceiling relative proportions in the total microbial counts were setup as special criteria for the individual microorganisms: for lactobacilli and eubacteria up to 100%, for streptococci 50%, for bifidobacteria 40%, for coryne- and propionibacteria 20%, for bacilli, anaerobic cocci and bacteroids 10%, for yeasts, staphylococci, ß-haemolytic strepto- and staphylococci not more than 1%. Different individual types of vaginal microflora in pregnant women have been described, various lactobacilli-containing types being the most frequent ones. These types are characterised by remarkable specificity and stability. A tight correlation between bacterioscopic diagnosis of BV and the increased number of viable BV-related microorganisms has been revealed.

2. The special criteria elaborated by us were introduced to diagnose imbalance in the vaginal microflora during pregnancy. We found that the vaginal microflora in patients with a threatened abortion can be characterised by a relatively high occurrence and predominance of certain opportunistic pathogens: at the moment of threatened abortion, an increase of beta haemolytic streptococci, bacteroids and anaerobic cocci was observed in VMf of some one third of the investigated women. These changes disappeared if the pregnancy lasted due to hormonal treatment and did not influence the outcome of pregnancy.

3. Some dynamic changes in VMf were noted during pregnancy: increase in the incidence and relative proportion of lactobacilli with a simultaneous decrease in the occurrence of bacterial vaginosis and opportunistic microorganisms. These dynamic microbiological findings show that some changes in the woman's organism during pregnancy as the hormonal status may probably help to stabilise the vaginal microbial ecology. Therefore, the treatment of bacterial vaginosis during pregnancy seems to be reasonable only in cases of repeatedly confirmed diagnosis.

4. We could observe that vaginal lactobacilli of pregnant women are not uniform as to their susceptibility to antibiotics, hence, it is difficult to predict their sensitivity patterns. Yet, most strains are susceptible to betalactams, macrolides and tetracyclines, but resistant to aztreonam and ofloxacin. This finding seems to be important in choosing lactobacilli-saving antibacterial treatment during pregnancy.

5. Our investigation revealed that both qualitative and quantitative characteristics, incl. predominance pattern of the vaginal microflora of a birth-giving mother are closely related to the corresponding data of the skin microbes of her newborn immediately after delivery. It can be supposed that the selective survival of cocci and low lactobacillar counts revealed by us in vaginal samples during delivery are associated with artificial medical interference, e.g. extensive repeated vulvar disinfection during labour. This altered predominance pattern is accordingly reflected in the newborn's initial microflora. We could not find extensive colonization by *Clostridium difficile* and GBS in the specially investigated pregnant Estonian women's delivery tract. Nor did any of the examined newborns harbour these microorganisms, which proves the reliability of the results obtained by studying the pregnant women.

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TUPE MIKROFLOORA RASEDUSE KORRAL JA SELLE ÜLEKANNE VASTSÜNDINULE

Kokkuvõte

Tupe normaalne mikrofloora on äärmiselt kompleksne ja keeruline süsteem, mille uurimisega on tegeldud juba üle saja aasta. Siiski on hulk probleeme veel lahendamata. On teada, et tupes võib leiduda suur hulk mitmesuguseid mikroorganisme ning iga naise tupefloora koostis on eripärane (Onderdonk and Wissemann, 1993; Larsen, 1993; Lee *et al.*, 1995). Iga üksiku mikroorganismi esinemine või puudumine ei ole mikrofloora kui terviku seisukohast eriti oluline. Seetõttu oleks vaja välja töötada metoodika, millega saaks hinnata mikrofloorat kui tervikut. Seedetrakti mikrofloora kohta on selline uudne metoodika hiljuti pakutud (Mikelsaar, 1992), tupe mikrofloora osas püüab seda lünka täita käesolev uurimus. Kuna tupe mikrofloora koostis on äärmiselt oluline mitte ainult naise, vaid ka tema laste tervise seisukohalt, siis on eriti tähtis uurida seda raseduse ajal. On teada, et ema tupefloora paneb olulise aluse tema lapse normaalse mikrofloora kujunemisele (Mikelsaar *et al.*, 1989; Ahtonen, 1994), kuid pole lõplikult selge, missugune tupefloora on vastsündinu jaoks soodsaim ja kuidas seda moodsate sünnitusabimeetoditega mitte kahjustada. Samal ajal seostatakse mitmesuguseid tupes leiduvaid oportunistlikke ja patogeenseid mikroorganisme intraamniaalsete, puerperaalsete ja vastsündinuinfektsioonide tekkega, samuti enneaegse sünnituse ja enneaegse lootekestade rebendiga (Gilbert *et al.*, 1995; Hillier *et al.*, 1995; McGregor *et al.*, 1995). Ometi ei tähenda nende mikroorganismide leid veel üheselt nimetatud patoloogiliste seisundite teket ning seetõttu oleks vaja uurida nii mikroorganismide omavahelisi kui ka makro- ja mikroorganismide vahelisi suhteid. Kuna mikrofloora dünaamilisi uuringuid raseduse vältel on tehtud väga vähe, siis pole teada, kas raseduse algul leitud muutused tupe mikroflooras kaovad sünnituse ajaks või vajavad sekkumist. Samuti pole teada, kuidas mõjutavad tupefloora koostist raseduse ajal mitmesugused ravimid (antibiootikumid, hormoonipreparaadid).

UURINGU EESMÄRK

I. Välja töötada meetodid tupe mikrofloora seisundi hindamiseks raseduse korral.

II. Kasutada väljatöötatud meetodeid:

1. tupe mikrofloora dünaamiliseks jälgimiseks raseduse vältel ning uurida mõningaid seda mõjutavaid faktoreid;
2. raseduse normaalse kulu ja pärast ähvardavat aborti püsima jäänud rasedusega naiste tupe mikrofloora võrdlemiseks;
3. erisuguse sünnituskuluga naiste tupe mikrofloora võrdlemiseks.

III. Kindlaks teha lapse võimalik koloniseerimine ema poolt sünnituse ajal:

1. normaalse mikrofloora esindajatega;
2. mõningate oportunistlike mikroobidega.

UURITAVAD JA MEETODID

Uuriti 138 rasedat naist ja 34 vastsündinut, kellel tehti kokku 391 tupe mikrofloora, 22 rektaalse mikrofloora ja 34 väliskuulmekäigu mikrofloora kvantitatiivset uuringut.

Rasedad jaotusid kuude gruppi. I–V grupi naisi uuriti Tartu Ülikooli Naistekliinikus, VI grupi naisi Indiana Ülikooli Haiglas USA-s. Tupe mikrofloora kvantitatiivsed dünaamilised uuringud tehti kokku 69 rasedal. 23 rasedat olid esmassünnitajad, kes ilmusid günekoloogi vastuvõtule enne 17. rasedusnädalat (I grupp); II grupp koosnes 19 ja III grupp 27 rasedast, kes pöördusid günekoloogi poole raseduse ähvardava katkemise tõttu enne 12. rasedusnädalat. I ja II grupi naiste tupe mikrofloorat uuriti raseduse vältel 4–7 korda, uuringuperioodid olid $\leq$10., 11.–16., 17.–22., 24.–26., 28.–30., 32.–34. ja 36.–38. rasedusnädalal, III grupi rasedate tupefloorat uuriti raseduse vältel kaks korda: ähvardava abordi ajal ja 32. rasedusnädalal. IV grupp koosnes 22 naisest, kellel uuriti tupe ja soole mikrofloorat semikvantitatiivselt raseduse 3. trimestril *C. difficile*, teiste klostriidide ja laktobatsillide suhtes. V grupp koosnes 30 ja VI grupp 17 rasedast, kelle tupest isoleeritud laktobatsillide tundlikkust uuriti antibiootikumide suhtes, V grupi rasedatel uuriti ka B-grupi streptokoki esinemist.

42-st I ja II grupi naisest õnnestus pärast sünnitust uurida 34 naise tupe mikrofloorat koos tema vastsündinu väliskõrva mikrofloora uurimisega. Neist 19 ema-laps-paari uuriti vahetult pärast sünnitust, 4 paari 1–12 tundi hiljem ning 11 paari 2–8 päeva hiljem.

Mikrofloora uurimiseks kasutati kvalitatiivset ja kvantitatiivset bakterioloogilist ning bakterioskoopilist meetodit. Bakterioloogiliselt määrati 10 aeroobset ja 9 anaeroobset mikroobigruppi ning uuriti mikroobitüvede antibiootikumitundlikkust. Bakterioskoopiliselt kasutati mikrofloora

kombineeritud hindamismeetodit, loendades mikroobide morfotüüpe ja registreerides võtmerakkude olemasolu.

UURIMUSE PEAMISED TULEMUSED

Tupe mikrofloora hindamiseks töötati välja spetsiaalne mikrobioloogiline metoodika, mis baseerub mikrofloora koostise kvalitatiivsel ja kvantitatiivsel määramisel külvide ja mikroskoopiaga.

Mikrofloora hindamiseks uuritavate rasedate gruppide tasemel kasutati järgmisi näitajaid: mikroobide üldhulk; üksikute mikroorganismide esinemissagedus; domineerivate mikroorganismide spekter; mikrofloora individuaalne tüüp. Mikrofloora hindamiseks üksiku tupeuuringu tasemel kasutati näitajaid: üksikute mikroobide protsent mikroflooras; mikrofloora seisund otsese mikroskoopilise uuringu alusel.

Leiti, et rasedatel naistel on võimalik eristada seitset tüüpi vaginaalset mikrofloorat. Igale naisele on iseloomulik individuaalne tupe mikrofloora tüüp, mis on raseduse vältel küllaltki püsiv ja mis taastub ka pärast antibiootikumravi.

Seitsmel naisel 42-st I ja II grupi rasedast leiti püsiv bakteriaalne vaginoos kogu raseduse vältel, lisaks neile leiti veel seitsmel enamikus uuringutes BV. Niisugune püsiv BV ühtis kahe meie kirjeldatud mikrofloora tüübiga, seega ei saa neid tüüpe pidada normaalseks. Üldse leiti bakteriaalset vaginoosi 42%-l naistest, kokku 31% uuringutest.

Tupe mikroflooras täheldati raseduse kasvades mõningaid spetsiifilisi muutusi: laktobatsillide esinemissagedus ja osatähtsus mikroflooras suurenes, seevastu bakteriaalse vaginoosi ja mõnede oportunistlike mikroobide esinemissagedus vähenes. Varem ilmnes laktobatsillide hulga suurenemise tendents raseduse ähvardava katkemise tõttu gestageenravi saanud naistel. Need mikrofloora dünaamilised muutused kinnitavad naise organismis raseduse ajal tekkivate ümberkõlastuste, sealhulgas hormonaalse seisundi muutuste suurt mõju normaalsele mikrofloorale.

Selgitati, et raseduse ähvardava katkemisega naiste tupe mikroflooras on sagedamini ja suuremas hulgas mõningaid oportunistlikke mikroorganisme. Osadel nimetatud naistest leiti esimeses uuringus, s.t. raseduse ähvardava katkemise perioodil ß-hemolüütilise streptokoki, bakteroidide ja/või anaeroobsete kokkide osatähtsuse suurenemist. Need muutused hiljem kadusid ega mõjutanud sünnitust. Viimaste sünnituseelsete tupefloora uuringute võrdlemisel ei leitud erinevusi erineva sünnituskuluga naistel.

Leiti tihe seos kahe kasutatud metoodika — bakterioloogilise ja bakterioskoopilise — abil saadud tulemuste vahel. Väga hea korrelatsioon mikroobide morfotüüpide ja võtmerakkude olemasolu vahel tupe mikroflooras

võimaldab kasutada viimaseid ainsa kriteeriumina bakteriaalse vaginoosi kiireks diagnoosimiseks.

Leiti, et laktobatsillide antibiootikumitundlikkus on eri tüvedel küllaltki erinev. Siiski oli enamik tüvesid tundlikud ß-laktaam-antibiootikumide. erütromütsiini ja doksütsükliini suhtes, kuid resistentsed ofloksatsiini ja aztreonaami suhtes.

Tehti kindlaks, et vastsündinu esmase mikrofloora koostis sõltub otseselt ema tupe mikrofloora kvalitatiivsest ja kvantitatiivsest koostisest sünnituse ajal. Sünnitaja genitaalide korduv desinfitseerimine sünnitusmajas vähendab küll tupemikroobide üldhulka, kuid mõjutab tugevasti mikrofloora koostist, sealhulgas vähendades selektiivselt laktobatsillide hulka ja mõjutades suhteliselt vähe oportunistlikke mikroobe. Niisugune muutunud mikrofloora seab ohtu lapse mikrofloora edasise normaalse kujunemise.

Meie poolt spetsiaalselt uuritud Eesti rasedate sünnitusteedes ei leitud B-grupi streptokokke; *Clostridium difficile*'t leiti vaid ühel juhul. Neid mikroobe ei leitud ka ühelgi uuritud vastsündinul, mis kinnitab rasedate uurimisel saadud tulemusi.

Tööst tulenevad praktilised soovitused:

1) enne ravi määramist uurida rasedat korduvalt bakteriaalse vaginoosi suhtes;
2) bakteriaalse vaginoosi kiireks diagnoosimiseks raviarsti poolt kasutada tupesisaldise mikroskoopiat võtmerakkude sedastamiseks;
3) kasutada tupe mikrofloora kvantitatiivset analüüsi selle koostise muutuse diagnoosimiseks komplitseeritud juhtudel, kui pole tegemist seksuaalselt ülekantavate haiguste, bakteriaalse vaginoosi ega kandidoosiga;
4) antibakteriaalse ravi ordineerimisel kasutada võimaluse korral aztreonaami või ofloksatsiini kui tupe laktofloorale ohutuid antibiootikume;
5) sünnitusteede puhastamisel asendada tugevatoimelised desinfektandid tavaliste pesemisvahenditega.

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PUBLICATIONS

I

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VAGINAL MICROFLORA DURING PREGNANCY

TUPE MIKROFLOORA RASEDUSE KORRAL

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Key words: vaginal microflora, pregnancy, bacterial vaginosis, threatened abortion

Summary

Rationale. We propose a method for evaluating the composition of vaginal microflora during pregnancy by estimating the pattern of microorganisms. Using this method, we studied changes in the vaginal microflora during pregnancy and their influence on pregnancy outcome.

Methods. The quantitative composition of vaginal microflora was investigated by cultures and by Gram-stained slides repeatedly (4-7 times) during pregnancy in 23 consecutive women and 19 women presenting early threatened abortion. A number of aerobic and anaerobic microorganisms were searched for and the predominant microorganisms (>10% of sum total count of microbes per sample) were determined.

Results. The vaginal microflora of the women could be divided into seven stable types. Lactobacilli-containing types were the most frequent in both groups; some types (corynéforms and cocci) were strongly correlated with bacterial vaginosis (BV) which was found in nearly one third of the samples. In women with an early threatened abortion, a predominance of ß-haemolytic staphylococci and ß-haemolytic streptococci was found more frequently in the first trimester; these disappeared during pregnancy.

Conclusions. No correlation was found between the quantitative composition of vaginal microflora prior to the 26th-36th week of gestation and the outcome of pregnancy.

Pakutakse välja metoodika tupe mikrofloora hindamiseks raseduse korral domineerivate mikroorganismide spektri alusel. Kasutades seda meetodit, uuriti muutusi tupe mikrofloooras raseduse erineva kulu korral ja nende mõju sünnituse kulule. Tupe mikrofloora kvantitatiivset koostist uuriti nii bakterioloogilise kui ka bakterioskoopilise meetodiga korduvalt (4...7 korda) 23-l esmassünnitajal ja 19-l rasedal, kes pöördusid günekoloogi poole raseduse ähvardava katkemise tõttu enne 12. rasedusnädalat. Bakterioloogiliselt määrati 20 erinevat mikroobigruppi ja igas uuritud mikrofloooras leiti domineerivad mikroorganismid (>10% mikrobide üldhulgast tampooni kohta). Leiti, et igal naisel on oma individuaalne tupe mikrofloora tüüp, nisuguseid tupe õrnestus eristada 7. Mõlemas grupis olid kõige sagedasemateks mitmesugused laktobatsillide kombinatsioonid teiste mikrobidega. Mõned tüübid (langesid kokku püsiva bakteriaalse vaginoosi esinemisega, mida leiti umbes ühes kolmandikus uuringutest. Raseduse ähvardava katkemisega naistel leiti esimesel trimestril sagedamini ß-hemolüütiliste streptokokkide ja ß-hemolüütiliste stafülokokkide domineerimist, mis hiljem kadus. Ei leitud korrelatsiooni sünnituse kulu ja tupe mikrofloora kvantitatiivse koostise vahel 36.-36. rasedusnädalal.

Introduction

It is well-known that the vaginal microbial ecology is a very complex system containing various aerobic, microaerophilic and anaerobic microorganisms [1, 2]. It is influenced by multiple endogenous and exogenous factors, which fluctuate periodically due to the conditions of the host, making evaluation a very complicated task [1]. During pregnancy the vaginal microflora has a tendency to change, becoming more lactic-acid-producing; opportunistic microorganisms rarely appear [3-5].

The intranatally-revealed imbalance of vaginal microflora has been associated with adverse pregnancy outcome such as premature labour, preterm delivery, premature ruptures of membranes and intrauterine growth retardation [6-11]. Various changes of the microflora, such as bacterial vaginosis, group-B streptococci, mycoplasmas and anaerobic microorganisms, have been associated with adverse pregnancy outcome [12-17]. However the investigators have not always considered the qualitative and the quantitative aspects of cervicovaginal microflora to be equally important. To our knowledge, there are no studies comparing the quantitative composition of vaginal microflora by microscopic and cultural examination during pregnancy.

Moreover, few data are available comparing reproductive tract microflora and the obstetric outcome of women who have recovered from an early threatened abortion [18-20]. Dynamic investigations of vaginal microflora have to determine whether the imbalance of microflora in early pregnancy can persist and jeopardize the obstetric outcome.

The aim of our study was to elaborate a method for the evaluation of vaginal microflora to enable comparison over the course of both normal pregnancy and in case of a threatened abortion, and to reveal how microflora composition influences the outcome of pregnancy.

Materials and Methods

Subjects. Two groups of pregnant women were investigated during a prospective study at Tartu Maternity Hospital (Table 1). Group I consisted of 23 pregnant women. Inclusion criteria for this group were: presenting before the 17th week of gestation, first expected delivery; body weight (in kg) before pregnancy less than body length (in cm) - 100 - 10%. Group II consisted of 19 consecutive women who were considered eligible for the study if they had been hospitalized owing to a threatened abortion before the 12th week of gestation. A threatened abortion was defined as multiple painful uterine contractions with or without vaginal bleeding which did not lead to abortion after treatment.

All the women were examined 4-7 times, with a total of 234 samples. The periods of sampling were: ≤10 weeks; 11th-16th, 17th-22nd; 24th-26th; 28th-30th; 32nd-34th, and 36th-38th weeks of gestation. In both groups a total of 117 quantitative investigations of vaginal microflora were made. Forty women out of 42 delivered live babies, one delivered a *foetus mortus* with multiple abnormalities and information about the delivery of one person is unavailable. Adverse pregnancy outcome (premature labour, preterm delivery, premature rupture of membranes) was noted in 11 women in both groups.

Specimens. Specimens for vaginal microflora investigation were taken from the lateral part of the internal region of the vagina. In order to maintain the viability of fastidious

Table I
Clinical data of pregnant women investigated

Pregnant women	N° of women	N° of investigations	Age range (mean)	N° of previous pregnancies: range (mean)	N° of women with		
					antimicrobial treatment	hormonal treatment	adverse pregnancy outcome
Group I	23	117	19-29 (22.9)	0-2 (0.3)	9*	0	11***
Group II	19	117	20-35 (26.7)	0-5 (2.2)	8*	19**	11***

* Nitrofurans for urinary tract infections (4 women of Group I; 4 women of Group II). Metronidazole for trichomoniasis (1:1). Clotrimazole (3:0) or Nystatin (0:1) for vaginal candidiasis. Sulphonamides (2:3). Ampicillin (0:1) or Oxacillin (0:1) for respiratory tract infections. All drugs were used as short courses during the 2nd and 3rd trimester of pregnancy.

** Turinal (Gedeon Richter, Hungary) contains 5 mg allylocstrenol. The hormonal therapy (5-15 mg daily) was started at arrival and stopped before the 20th week of gestation.

*** Premature labour (2 cases in Group I, 1 case in Group II), preterm delivery (1:2), premature rupture of membranes (5:5), combination of two adverse outcomes (3:3).

microorganisms, bloodthioglycollateagar coated cottonwool swabs were used. The swabs were put into tubes containing carbon dioxide sealed with rubber stoppers. Specimens were sent to the laboratory within two hours of collection.

Media, cultivation and identification. The vaginal swabs were shaken in 2 ml of prereduced phosphate buffer under a gentle stream of oxygenfree CO_2. Dilutions 10^{-1}-10^{-8} (0.01 ml) of the bacterial suspension were then seeded into prereduced bloodthioglycollateagar medium handled as modified rolltubes for anaerobic microorganisms [21]. The material was also seeded onto freshly-prepared bloodagar with 5% human blood, onto lactobacilli- and streptococci-selective MRS4 agar [22], and onto Endo and Sabouraud media. The blood agar and Endo and Sabouraud media were incubated aerobically at 37°C and examined after 48-72 h. The MRS4 medium was incubated in 10% CO_2 at 37°C for 72 h and the roll-tubes at 37°C for 72-120 h.

All aerobes and facultative microorganisms were identified using standard methods [23]. The anaerobes were presumptively identified by colony and cellular morphology in Gram stain [24] after their aerotolerance was tested on blood agar and MRS2 [22]. The lactobacilli were identified on the basis of colony and cellular morphology, and negative catalase production [22].

For the detection of bacterial vaginosis, Gramstained slides were made from the first dilution of the material. The slides were evaluated using the scoring system of R. P. Nugent et al. [25]. The presence of clue cells was also recorded.

Statistical methods. The data were analysed using the Wilcoxon rank test, Student's *t*-test and correlation analysis. Regression analysis was performed to reveal dynamic changes in the relative proportion of different microorganisms during pregnancy [26].

Results

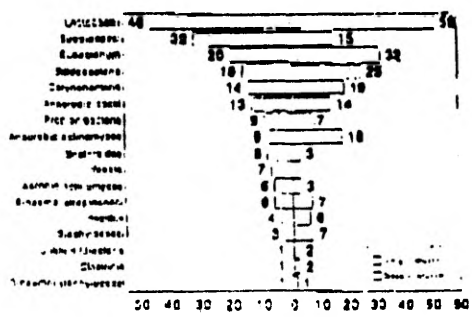
Examination of vaginal microflora by Gram stained slides. We found bacterial vaginosis (BV) in 36.2% of the samples from Group I and in 26.5% of the samples from Group II ($t = 1.67$, $P < 0.05$). BV was noted in 11 (47.8%) women from Group I and in nine (47.4%) women from Group II. Twelve women (nine in Group I and three in Group II) had a picture compatible with BV in all or most of the samples taken.

We could not detect any differences between the two groups of women studied ($P > 0.05$) nor any correlation of BV with adverse pregnancy outcome: five cases of BV in the last investigation ($P > 0.05$) were found both from 22 women with adverse and from 20 women with normal pregnancy outcome.

Vaginal microorganisms isolated in pregnancy. In Group I, the counts of microorganisms ranged from 0-7.6 log CFU/swab with a mean of 6.7 and in Group II from 2.2-7.7 log CFU/swab with a mean of 6.7. Hence the total counts of microbes were the same in both groups of women.

Various aerobic and anaerobic microorganisms were isolated in 232 cases out of the 234 samples analysed. No organisms were detected in two samples. Twenty different groups of microorganisms were isolated from both groups of women and none of them occurred in all women. We found some differences in the number of genera and groups of microorganisms isolated in the groups of women under investigation. In Group I, we were able to find 0-8 different microbes per individual sample (mean 3.6) and in Group II 1-8 (mean 4.7; $P < 0.05$). Lactobacilli, streptococci, bacilli and corynebacteria were the most frequent microorganisms in both groups of pregnant women.

Figure 1
N°of samples with predominance of selected microbe



The composition of vaginal microflora. To get a better idea of the quantities of different microorganisms making up the vaginal microflora, their relative distribution was calculated as a percentage of the sum total count of microbes in each sample. This approach made it possible to distinguish between the predominant and subordinate microbes in each sample. Microbes were considered predominant if they made up more than 10% of a microflora population, that is to say if their count did not differ from the total count more than one logarithm.

The ranks of different microorganisms were calculated according to their frequency of predominance within the groups. No significant differences were noticed between the ranks of Groups I and II ($Z = 0.87$, $P > 0.05$). In both groups, lactobacilli most frequently predominated (Figure 1). No predominance of *Veillonella*, *Sarcina* or coliforms was observed at any time.

The relative proportion of about half of the microbes (lactobacilli, streptococci, staphylococci, anaerobic cocci, corynebacteria, bacilli, *Eubacterium*, propionibacteria, *Actinomyces*, bifidobacteria.) could be as high as 100%, *i.e.* they could occur in the vaginal microflora as a single population. In contrast, the maximum proportion of *Veillonella* did not exceed 0.1%, that of *Sarcina* was always less than 0.2%, and that of coliforms was less than 2.6%.

Table 2
Occurrence of adverse pregnancy outcome and changes in predominance pattern of vaginal microflora at final examination before delivery

Opportunistic pathogens displaying increase as proportion of vaginal microflora	Adverse pregnancy outcome (N°=22)		Normal pregnancy outcome (N° = 20)	
	Percentage of total microbe count	N° of cases	Percentage of total microbe count	N° of cases
Bacteroides	94.97	2	16	1
Anaerobic cocci	30	1	16,11	2
Corynebacterium	71	1	25, 38, 41, 74, 99	5
Actinomyces	48, 60, 95, 98, 17	5	50	1
Bacillus	100, 30	2		0
Staphylococcus	67.100	2	66,100	2
β-haemolytic streptococci				
yeasts	11	1		0
Total N° of women with changes in vaginal microflora		12*		11*

* $P > 0.05$

Table 3
Types of microflora by predominance pattern

Type (stable predominance pattern)	N° of women			Persistent BV by Gram stain		
	Grl	Grli	Total	Grl	Grli	Total
1. Lactobacilli	1	2	3	0	0	0
2. Lactobacilli + streptococci	4	3	7	1	0	1
3. Lactobacilli + anaerobic coryneforms	2	3	5	0	0	0
4. Lactobacilli + *Eubacterium*	3	3	6	0	0	0
5. Eubacterium	3	2	5	0	2	2
6. Coryneforms	6	5	11	5	3	8
7. Cocci	2	1	3	2	0	2
Type not determined (varying microflora)	2	0	2	1	0	1

Stable BV is in strong positive correlation with types 5 and 7 ($r = 0.67$, $P < 0.01$)

On examining the vaginal microflora immediately before delivery, we were unable to detect any correlation between the predominance of opportunistic pathogens and adverse pregnancy outcome ($P > 0.05$, Table 2). The most frequent opportunists in both cases (with and without adverse pregnancy outcome) were a number of coryneforms, and a predominance of bacteroides, cocci, bacilli and yeasts could be also noted.

Types of microflora. On the basis of the occurrence of stable predominant bacteria in vaginal microflora, the women could be classified into seven types (Table 3). Only in

Discussion

Two different methods (microscopic examination of the vaginal smears and quantitative microbe cultivation) were used to investigate possible changes in the vaginal microflora of the women during pregnancy.

A wide variety of microorganisms in different combinations was isolated from the vaginas of the pregnant women, which is in line with results obtained by other investigators [27, 28]. This variety has always made the evaluation of vaginal microflora difficult [3-5]. To overcome this difficulty, we aimed to determine the predominant microbes in the microflora. We found a number of microbes that may potentially predominate (Figure 1) but not all of them constitute a stable part of the microflora. Certain microbes (staphylococci, bacilli, *Bacteroides*, β-haemolytic staphylococci) can occur in very large numbers in a few cases only but are never present as stable elements over the entire course of pregnancy. Thus their predominance may be seen as a temporary imbalance of the ecosystem. Our quantitative bacteriological results were in good accordance with the data of examination of direct smears of vaginal samples. We therefore suggest that vaginal microflora should first be evaluated by microscopic examination and only in complicated cases (the absence of trichomoniasis, candidiasis and bacterial vaginosis) by quantitative evaluation to reveal the predominance patterns of microorganisms.

On the basis of the stable predominant microorganisms we were able to identify seven types of vaginal microflora in pregnancy. This finding is in accordance with Onderdonk et al, who hypothesized that there is probably no one type of vaginal microflora which is 'normal" for all women [1]. It is especially interesting to note that not even antimicrobial treatment altered the individual type of microflora; the stable predominance pattern was re-established at 2-7 weeks after treatment, a finding in good correlation with the data of Grossman et al [29]. Stability is apparently associated with individual receptor pattern of the host's cells [30]. Fluctuations in the composition of the microflora may be due to the open nature of the vaginal ecosystem, which is periodically in contact with external microflora. Stability is also a characteristic of gastrointestinal microflora, which has been found to be stable for as long as thirteen years [30]. The occurrence of certain 'types" (coryneforms, cocci) was in good correlation with persistent bacterial vaginosis.

The vaginal microflora of two groups of women during the course of pregnancy was evaluated comparatively using the methods developed and subsequently related to the outcome of pregnancy. The first group of women consisted of consecutive primiparae with normal, or slightly lower than normal, body weight. The second group consisted of women who had been hospitalized before the 12th week of pregnancy owing to a threatened abortion, and whose pregnancy was sustained as a result of medical treatment and completed successfully. Examination of the two groups of pregnant women did not start in the same week of gestation because most of the Group II women had consulted the gynaecologist earlier because of a threatened abortion. However in both groups the first examination fell in either the first (< 10th week) or the second (11th-16th weeks) sampling period. Among both groups of women there were some individuals who had been treated with antimicrobials for urinary or respiratory tract infections, trichomoniasis or vaginal candidiasis. BV was registered with almost identical frequency in both groups. The groups under investigation were therefore substantially similar populations of pregnant Estonian women. The main difference lay in the presence or absence of early threatened abortion. In both groups a nearly equal

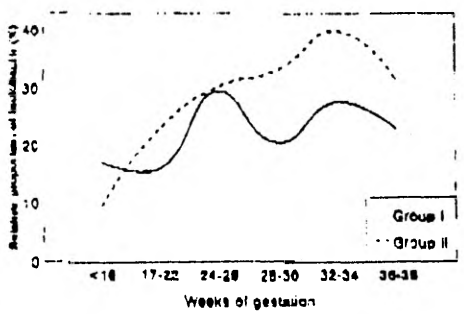
two cases were we unable to determine the type of the vaginal microflora. The lactobacilli-containing types were the most frequent ones in both groups (a total of 21 cases) yet the pure lactobacillus-type was found in only three women. In most cases a predominance of lactobacilli was accompanied by high counts of *Eubacterium*, streptococci or coryneforms (including *Corynebacterium*, *Propionibacterium*, *Bifidobacterium* and *Actinomyces*). The microbes of greatest pathogenic potential, such as coliforms, clostridia, bacteroides and ß-haemolytic streptococci, did not form a stable part of the microflora and were evident in only a few samples.

We found that the persistent BV diagnosed by Gram-stained slides had a good correlation with certain types of microflora, such as the predominance of aerobic and anaerobic coryneforms and also of cocci ($r=0.67$, $P < 0.01$).

There were no significant differences in the occurrence of these types of microflora in the two groups of women studied ($P > 0.05$).

Changes in the vaginal microflora during pregnancy. The predominant microbes in the individual vaginal ecosystem of some women appeared to be the same in all samples taken during pregnancy but on average they were the same in 62.3% samples in Group I and in 62.1% samples in Group II. Thus the microflora of each individual appeared to be significantly constant during pregnancy. Even after antimicrobial treatment (Table 1), the predominance pattern of microflora remained unchanged in all women for the next investigation, performed 2-7 weeks after the completion of therapy.

Figure 2
Average relative proportion of lactobacilli in vaginal microflora during different periods of pregnancy



During the follow-up, some dynamic changes were noticed in certain microorganisms. With regard to lactobacilli, the microorganisms which play the main protective role in the vagina, we observed an increase in their relative proportion in the total microbe count in both groups ($r=0.54$ and $r=0.83$, respectively). This increase started earlier in the Turinal-treated Group II (Figure 2).

The relative proportion of some other constituents of the vaginal ecosystem also showed a tendency to change. The relative quantity of staphylococci increased during pregnancy in both groups ($r=0.62$ and $r=0.63$, respectively) whereas the quantities of *Propionibacteria* ($r=-0.82$ and $r=-0.66$) and anaerobic cocci ($r=-0.56$ and $r=-0.85$) decreased. *Bacteroides* ($r=-0.51$) and *Bifidobacteria* ($r=-0.88$) decreased in Group II.

With regard to opportunistic microbes, in Group II certain differences were observed in the predominance of such opportunistic bacteria as ß-haemolytic staphylococci ($r=-0.70$) and ß-haemolytic streptococci ($r=-0.89$) in the first trimester of pregnancy, in comparison with the second and third trimesters. On following four separate patients who showed predominance of ß-haemolytic streptococci at the time of threatened abortion, we noticed that later these microorganisms completely disappeared (their relative proportions in microflora at the first ⇒ second ⇒ third estimation of different women being: 81.4%⇒33%⇒0%; 16.5% ⇒ 0% ⇒ 0%; 45% ⇒ 0% ⇒ 0%; 48% ⇒ 0% ⇒ 0%).

number of women with adverse pregnancy outcome was registered. This which was compared against data from the dynamic investigation of the women's vaginal microflora.

We were able to detect some differences in the vaginal microflora of the two groups investigated. The increase in lactobacilli counts in the microflora started earlier in Group II, evidently as a result of hormonal treatment with Turinal. The predominance of some opportunistic pathogens, revealed at the time of threatened abortion, quickly disappeared during Turinal therapy. This is probably explained by data showing that oestrogens and progesterone will change the adhesion capacity of microbes to eucaryotic cells [31].

We could not confirm the correlation between BV and the adverse obstetric outcome which has been described by others [32, 33]. Nor could we find any correlation between the predominance of opportunistic pathogens in the last examination before delivery and adverse pregnancy outcome. The larger numbers of opportunistic microorganisms found at the time of threatened abortion disappeared later and did not influence the eventual obstetric outcome. It seems that comparison of changes in the vaginal microflora with pregnancy outcome requires a study of the quantitative composition of vaginal microflora in larger numbers of women just before delivery. Germain et al [6] have found, in an investigation of thousands of women without quantitative analysis, that some vaginal microbes have an additive or synergistic impact on obstetric outcome and have recognized the necessity for the quantitative assessment of microflora. In this paper, we propose one method for carrying out such an assessment.

We found that vaginal ecosystem tends to remain stable, as is revealed in the persistent predominance pattern of its microflora; that the ecosystem a tendency to become more lactic-acid producing as gestation advances; and that there is no adverse influence of early threatened abortion on the obstetric outcome.

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II

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BACTERIAL VAGINOSIS DURING PREGNANCY

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SUMMARY

We investigated the vaginal microflora of 42 women repeatedly (4-7 times) during pregnancy (in the period between 6 and 38 weeks of gestation). Dilutions of the material were inoculated into different media and incubated aerobically and anaerobically. Subsequently the total count of microorganisms per swab and the relative percentage of lactobacilli in the total microbial count of the vagina were calculated. From the first dilution of the material also a Gram-stained smear was made which was evaluated using the scoring system of *Nugent* et al. (1991) for diagnosing bacterial vaginosis (BV). Also the presence of "clue cells" was registered.

We found BV in 31.3% of the samples. At least one episode of BV during pregnancy occurred in 20 women out of 42, seven of them had BV in all the samples. "Clue cells" were found in 68 out of 72 samples with BV and in two cases out of 114 intermediate microflora.

Lactobacilli were found in 39 out of 42 women and in 139 out of 229 samples. Their median relative amount in the microflora was similar in case of normal and intermediate vaginal flora whereas it was 0 in all the scores of BV. The incidence and relative amount of lactobacilli in the vaginal microflora increased during pregnancy, at the same time the incidence of BV by Gram-stain decreased. A dynamic survey of pregnant women can provide a model for studying the microbial ecology of BV.

INTRODUCTION

Bacterial vaginosis (BV) is found to be quite common among pregnant women (*Cristiano* et al., 1989; *Kurki* et al., 1992; *McGregor* et al., 1990; *Thomason* et al., 1991). It has been associated with several pregnancy complications (*Baron* and *Finegold*, 1990; *Hoyme*, 1989; *Kurki* et al., 1992; *Mardh*, 1991; *McGregor* et al., 1990; *Nugent* et al., 1991) and the altered vaginal microflora may induce changes in the microflora formation of the new-born (*Lundequist* et al., 1985).

In BV the absence or low numbers of lactobacilli is reported (*Spiegel*, 1991; *Thomason* et al., 1991). However, it is not known whether the

Table 1: Characteristics of the pregnant women investigated

Study group	No.	Age (range and mean)	Number of previous pregnancies (range and mean)	Number of women with anti-bacterial treatment	Number of women with hormonal treatment	Adverse pregnancy outcome[3] (%)
Group I	23	19-29 (22.9)	0-2 (0.3)	9[1]	0	11/23 (47.8)
Group II	19	20-35 (26.7)	0-5 (2.2)	8[1]	19[2]	11/19 (57.9)

[1] Nitrofurans for urinary tract infections (4 women of Group I and 4 women of Group II), Metronidazole for trichomoniasis (1 and 1), Clotrimazole (3 and 0) or Nystatin (0 and 1) for vaginal candidiasis, Sulphonamides (2 and 3), Ampicillin (0 and 1) or Oxacillin (0 and 1) for respiratory tract infections.

[2] Turinal (Gedeon Richter, Hungary), contains 5 mg allyloestrenol. The hormonal therapy (5-15 mg daily) was stopped before the 20th week of gestation.

[3] Threatened prematurity, preterm delivery and/or premature rupture of membranes.

changes in counts of lactobacilli are directly correlated with the incidence of BV. Several investigators have described the increase in vaginal lactobacilli before birthgiving (*Galask*, 1988; *Redondo-Lopez* et al., 1990). At the same time there are very few dynamic studies of BV (*Platz-Christensen* et al., 1993) and no studies comparing these two indicators dynamically during pregnancy. Also we could not find any investigations comparing by cultivation the relative percentage of lactobacilli in the microflora and by Gram-stained slides in the incidence of BV.

The aim of the present survey was to study the occurrence and dynamics of BV during pregnancy and to compare it with the presence and amount of lactobacilli.

MATERIAL AND METHODS

Two groups of pregnant women were investigated during a prospective study at the Tartu Maternity Hospital (Table 1). The first group consisted of 23 consecutive pregnant women presenting before the 17th week of gestation who had never delivered before (Group I), and the second group of 19 consecutive women presenting with a threatened abortion before the 12th week of gestation (Group II). Each woman was investigated 4-7 times; altogether 229 investigations were performed. The periods of sampling were ≤10 weeks, 11th-16th, 17th-22nd, 24th-26th, 28th-30th, 32nd-34th and 36th-38th weeks of gestation, during ordinary visits to gynaecologist. Forty out of 42 women delivered live babies, one delivered a foetus mortus with multiple abnormalities and we lack information about the course of delivery of one person.

Specimens were taken from the lateral side of the internal part of the vagina. To maintain the viability of fastidious microorganisms blood-thioglycollate-agar-coated cottonwool swabs were used. The swabs were put

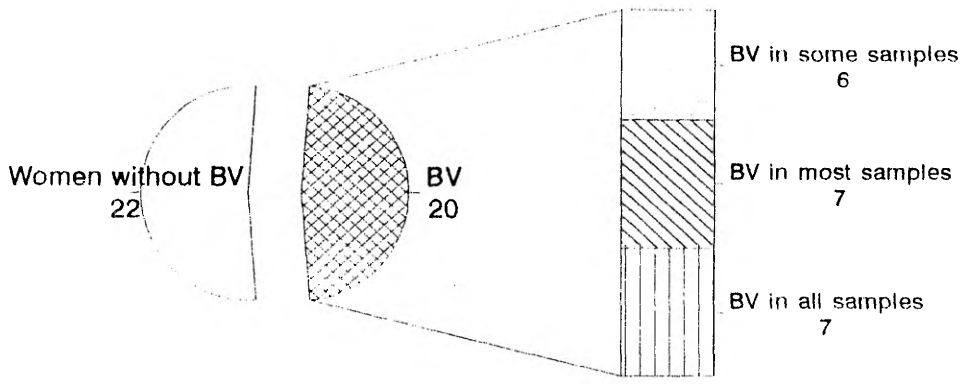

Figure 1: Occurrence of BV among pregnant women.

into tubes containing carbon dioxide sealed with rubber stoppers. The specimens were sent to the laboratory within 2 hours of collection.

Before inoculating the plates, the swabs were shaken in 2 ml of pre-reduced phosphate buffer under a gentle stream of oxygen-free CO_2. Dilutions 10^{-1}-10^{-5} (0.1 ml) of the bacterial suspension were then inoculated into pre-reduced blood-thioglycollate-agar- medium handled as modified roll-tubes for anaerobic microorganisms (*Mikelsaar* et al., 1984). The material was also inoculated onto freshly prepared blood-agar with 5% human blood, onto lactobacilli and streptococci selective MRS-4 agar (*Lenzner* et al., 1984), and onto Endo and Sabouraud media. The blood agar and the Endo and Sabouraud media were incubated aerobically at 37°C and examined 48-72 h later. The MRS-4 medium was incubated at 37°C in 10% CO_2 for 72 h and the roll tubes at 37°C for 72-120 h.

The lactobacilli were identified on the basis of colony and cellular morphology and negative catalase production (*Lenzner* et al., 1984). In case of each separate sample we calculated the total count of microorganisms per swab and the relative percentage (%) of lactobacilli in the total count of microbes of the vagina.

From the first dilution of the material also a Gram-stained smear was made for diagnosing BV. The slides were read using the scoring system of *Nugent* et al. (1991): large Gram-positive rods, small Gram-negative and -variable rods and curved Gram-variable rods have to be counted in 3 different oil-immersion fields and the criterion for BV is a score of 7 or higher; a score between 4 and 6 is considered intermediate, and a score between 0 and 3 normal.

Also the presence of "clue cells" was registered.

Data were analysed by regression analysis.

RESULTS

We found bacterial vaginosis in 72 (31.3%) out of 229 investigations. At least one episode of BV during pregnancy had occurred in 20 (47.6%) women out of 42 (Figure 1). Fourteen of them had BV in most of the sam-

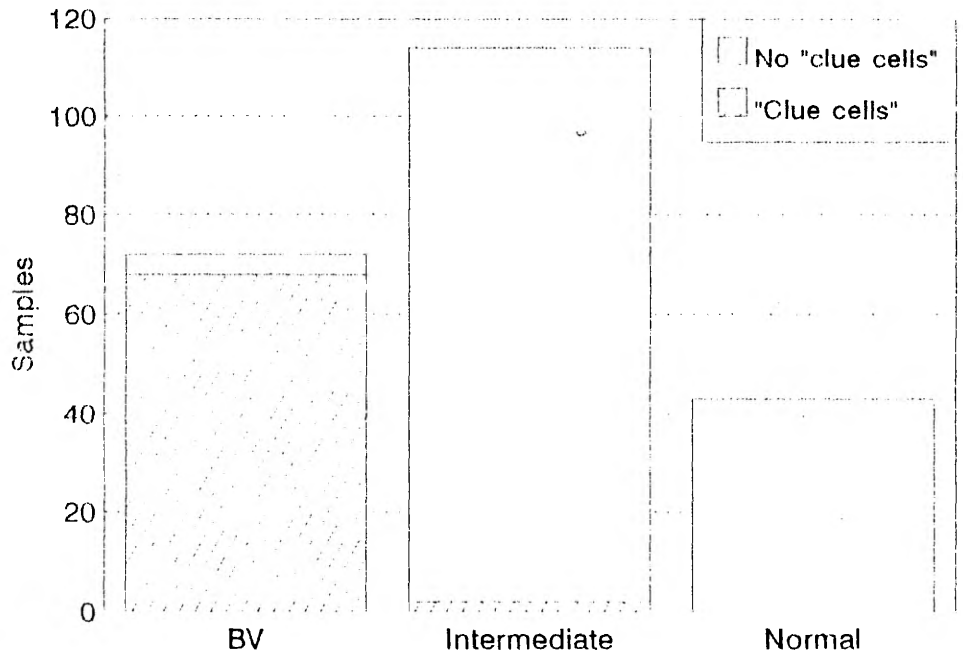

Figure 2: Occurrence of "clue cells" in different vaginal microfloras.

ples, in 7 of them it was found in all the samples. In 22 women BV was never revealed.

Nearly half (49.8%) of all the investigations showed the intermediate vaginal microflora, the normal microflora being found only in 18.8% of the samples. Not a single woman was found to harbour a normal microflora (score 0 - 3) during their complete pregnancy, everyone of those 22 without BV had at least one episode of intermediate vaginal microflora.

"Clue cells" were found in 68 out of 72 samples with BV (Figure 2). They were also found in two cases out of 114 in the intermediate and never once in the normal microflora ($r = 0.94$).

We could find little difference between two groups studied; in women with threatening abortion BV could be detected less frequently (Table 2).

Table 2: Occurrence of BV in the different groups

Study group	Occurrence of BV (%)			
	Total % of samples	No. of women with BV in the first sample	No. of women with BV in the last sample	Stable BV[1]
I	36.2	8/23 (34.8)	8/23 (34.8)	7/23 (30.4)
II	26.5	8/19 (42.1)	2/19 (10.5)	5/19 (26.3)

[1] in more than half of samples.

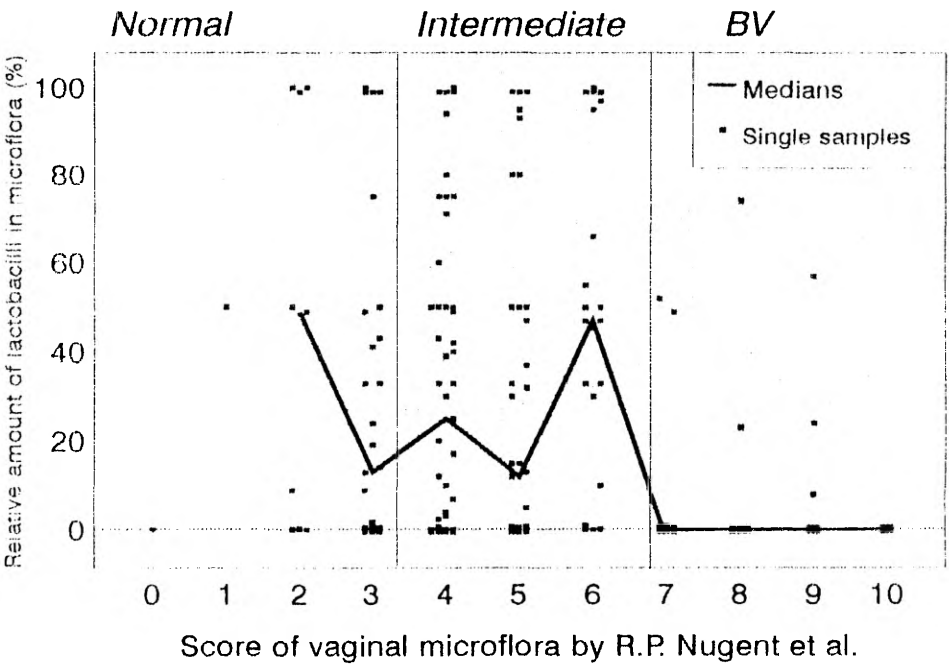

Figure 3: Relative amounts of lactobacilli in different vaginal microfloras.

Lactobacilli were found in 39 (92.8%) out of 42 women and in 139 (60.7%) out of 229 samples. Their median relative amount in the microflora did not differ in cases of the normal and intermediate vaginal flora, whereas it was 0 in all scores of BV (Figure 3).

The incidence and relative amount of lactobacilli in the vaginal microflora increased during pregnancy ($r = 0.90$, $p = 0.005$; Figure 4). At the same time the incidence of BV by Gram-stain decreased ($r = -0.62$, $p = 0.13$). Also the mean score of vaginal flora decreased during pregnancy (from 5.2 to 4.7).

DISCUSSION

We could detect BV quite frequently (in 31.3% of the samples) in the pregnant women that we examined. The incidence of BV corresponds to the data provided by other authors who have found BV in 10 to 26% of pregnant women (*Cristiano* et al., 1989; *Kurki* et al., 1992; *McGregor* et al., 1990; *Platz-Christensen* et al., 1993; *Thomason* et al., 1991). Our study confirms the previous findings that the presence of the "clue cells" is really a good marker of BV which allows to diagnose BV rapidly without counting microbial morphotypes.

At the same time the total number of women harbouring BV in at least one sample during pregnancy was very high (20 out of 42 i.e. 47.6%). Our dynamical investigation showed

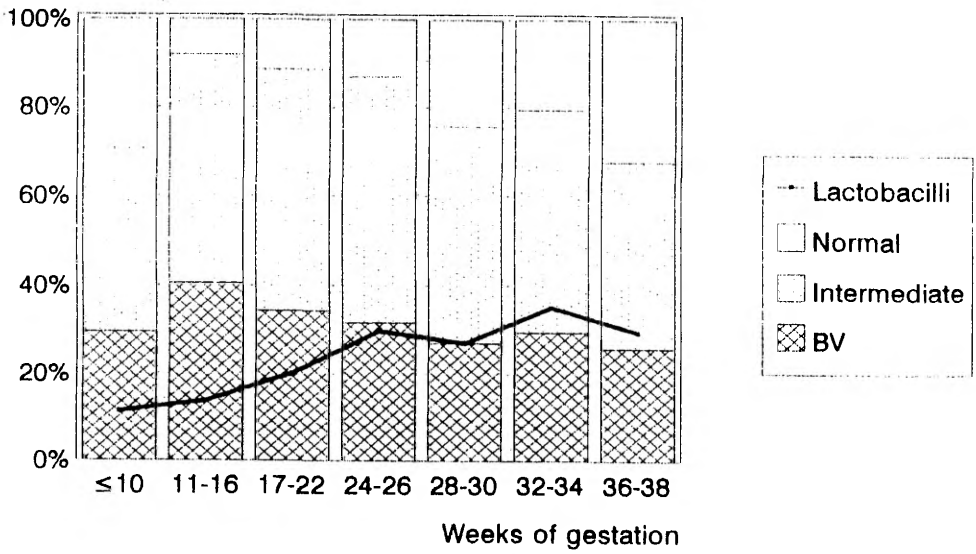

Figure 4: Dynamics of the relative amount of lactobacilli and occurrence of BV during pregnancy.

that in some women BV was unstable, whereas others had it throughout their pregnancy. The instability of BV has been previously described but mainly in nonpregnant women (*Bump* et al., 1988; *Platz-Cristensen* et al., 1993; *Livengood* et al., 1990; *Piot* et al., 1983). Consequently, the treatment of BV during pregnancy can be postponed till the diagnosis of BV is repeatedly confirmed.

In Group II the BV was somewhat less frequent, especially at the end of pregnancy. This may be connected with their hormonal therapy which has been shown to influence the microbial types and population levels in the vagina (*Redondo-Lopez*, 1990).

Lactobacilli were the frequent colonisers of pregnant women. We found that their relative amount in the microflora increased towards the end of pregnancy. This finding is in good correlation with the complementary decrease of incidence of BV in the same women. Seemingly the colonisation of the vagina by lactobacilli really intervenes with the incidence of BV, apparently by improving the microbial ecology of the vagina.

We could not find differences between the relative amount of viable lactobacilli in case of the normal and the intermediate vaginal floras. The possible explanation may be that the "lactobacillus-morphotype" in Gram-stained slides partially includes also some other Gram-positive rods eubacteria, bifidobacteria, bacilli, and actinomyces. These microbes may help to perform the colonisation resistance of vaginal microflora. According to our survey it seems that the total amount of Gram-positive rods gradually decreases up to the point when in case of BV both lactobacilli and all the other Gram-positive rods disappear. So only by bacterioscopic studies it would not be possible to determine the real composition of Gram-positive flora and make conclusions about the interrelations between the persistence of lactic acid microflora and incidence of BV.

Consequently, pregnancy serves as a useful natural model for revealing dynamical ecological changes in vaginal microflora.

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III

Biology of the Neonate **1996, 69: 30–35**

Original Paper

Biology of the Neonate

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Transmission of Mother's Microflora to the Newborn at Birth

Key Words
Microflora
Vagina
Delivery
Newborn
Colonization

Abstract
Our aim was to study the initial microbial colonization of the newborns by comparing it with their mothers' vaginal microflora. Nineteen mother-newborn pairs were examined at delivery. We found a close association, both qualitative and quantitative, between the individually different microflora of a mother's vagina and that of her newborn. The degree of contamination of the newborn significantly correlated with the counts of microorganisms found in the vagina of mothers. In 85% of investigated individual mother-newborn pairs we revealed similar predominant microorganisms. There were no cases of the mothers and their newborns harbouring similar potentially pathogenic prevailing microorganisms.

Introduction

The sudden passage of a sterile fetus through a complex microbial world persistent in vagina makes it crucially important from the point of view of the child's health what kind of microbes contaminate it first and begin to multiply on it [1–3]. Vaginally delivered newborns evidently acquire their initial microorganisms from vagina, gastrointestinal tract and skin of their mothers [4–6]. Besides the beneficial microorganisms of the indigenous microflora (like mainly lactic acid bacteria), the newborn usually gets also from its mother some opportunistic pathogens, such as β-haemolytic *Streptococci*, coliforms and *Clostridia* [3, 7–9]. Unfortunately, there are no data showing what microecological relations exist between the beneficial and opportunistic microorganisms in healthy newborns at the moment of delivery.

Several authors have presumed that the modern obstetrical practice during birthgiving (including treatment of the genital tract with disinfectants) may alter the quantitative composition of mother's microflora and cause a delayed and deficient colonization of the neonate by indigenous microflora [1, 5]. However, only a few investigators have compared the predominance patterns of a mother's vaginal microflora with her newborn's microbiota immediately after birth [3].

The aim of the study was to investigate the initial microbial colonization of a newborn

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and compare its qualitative and quantitative parameters with those of its mother's vaginal microflora during birthgiving.

Material and Methods

Subjects

In Tartu University Maternity Hospital, Tartu, Estonia, 19 consecutive mothers (aged 19–35) hospitalized for delivery and their babies were recruited into study. The infants were full-term and vaginally delivered, in 5 cases PROM was observed. At admission and shortly before delivery, vaginal and perineal douching application with a chlorhexidine solution (0.05%) was performed. All mother-newborn pairs were examined immediately after delivery.

Specimens

For newborns, the specimens were obtained from the external ear canal. For the mothers, the material was taken from the lateral side of the internal third part of the vagina. To maintain the viability of fastidious microorganisms, the specimens were collected using blood-thioglycollate-agar-coated cotton-wool swabs [10]. The swabs were put into tubes containing carbon dioxide and sealed with rubber stoppers. The specimens were sent to the laboratory within 2 h of collection.

Culture of the Specimen

The swabs were shaken in 2 ml of prereduced phosphate buffer (pH 7.2) under a gentle stream of oxygen-free CO_2. Serial dilutions 10^{-1}... 10^{-5} of the material were prepared. The dilutions (0.01 ml) were subsequently seeded into different media, such as the prereduced blood-thioglycollate-agar medium, handled as modified roll tubes for anaerobic microorganisms [11], freshly prepared blood agar with 5% human blood for aerobic microorganisms, lactobacilli- and streptococci-selective MRS-4 agar [12], Endo agar for coliforms, and Sabouraud agar for yeasts. The blood agar, Endo and Sabouraud media were incubated aerobically at 37°C and examined after 48–72 h. The MRS-4 medium was incubated in 10% CO_2 for 72 h and the roll tubes at 37°C for 72–120 h.

Identification of Isolates and Enumeration of Microbes

All the aerobes and facultative microorganisms were identified by using standard methods up to the genus level. The anaerobes were identified on the basis of their colony and cellular morphology by Gram stain [13, 14], and their absence of aerotolerance on blood agar and MRS-2 [12]. As a result, we identified Gram-positive anaerobic rods as *Bifido-*, *Propioni-* and *Eubacteria*, or *Actinomyces*, Gram-positive anaerobic cocci as *Pepto-* or *Peptostreptococci*, Gram-negative anaerobic cocci as *Veillonella* and Gram-negative anaerobic rods as *Bacteroides* or *Fusobacteria*. *Lactobacilli* were identified on the basis of their colony and cellular morphology and absence of catalase production [12]. We were unable to identify 4 of the isolated microbes and they were described as aerobic Gram-positive or Gram-negative coccobacilli. In each sample, the total count of microorganisms per swab was calculated. The density of bacterial growth was characterized by 2°: high and low, i.e. either more or less than 100 CFU/swab. The relative distribution of microorganisms was calculated as percentage of the total count of microbes. For each microbiocenosis in which the density of bacterial growth was high, the predominance pattern was determined by differentiating the predominant and subordinate microbes. We considered as predominant such microbes whose relative proportion in the total count of microorganisms exceeded 10%.

Results

Number of Microorganisms

Various aerobic and anaerobic microorganisms were isolated from all the mothers studied and from 15 babies. In 4 babies we could not detect any of the microorganisms under examination. The distribution of microbial counts in the external ear canal of newborns resembled their mothers' vaginal pattern: most of them – 84% of mothers and 74% of newborns – harboured high numbers of microorganisms (table 1). When the density of microbes in the maternal vagina was low, the infants were never heavily colonized.

Occurrence and Predominance of Different Microorganisms

We isolated 16 different groups of microorganisms from mothers and 13 from newborns (fig. 1). *Streptococci* were the most frequent microorganisms both in the mothers' vagina

Table 1. Total counts of microorganisms at delivery

Count of microbes (CFU/swab)	Number (%) of individuals		
	mother and newborn	mother only	newborn only
<100 (low density)	2 (10.5)	0	4 (21.1)
>100 (high density)	13 (68.4)	4 (21.1)	0

(47.4%) and the newborns' ear canal (36.8%). Various individually different combinations of up to 8 microbes were observed per sample in mothers and up to 6 microbes per sample in babies. We found that all the microorganisms detected in the ear of a child were present in the samples taken from its mother's perineum, but not all mothers' microbes colonized their newborn.

Comparing the predominance patterns of the microorganisms in the vaginal microflora of mothers and their children, we found *Streptococci* to be the most frequent predominant microbes: in 6 mothers and 5 newborns (fig. 1). As regards opportunistic microorganisms, we observed that yeasts never predominated either in mothers or in their newborns. β-haemolytic *Staphylococci* occurred among the predominant microbes only in unpaired samples (1 mother and 1 newborn), thus comprising less than 25% of the total count of microbes. β-haemolytic *Streptococci* predominated only in 1 mother (20% of the total count) and never in newborns. We could not find any difference in predominance patterns of the mothers' vaginal or babies' ear canal microorganisms between cases with and without PROM.

Comparison of the Predominant Microorganisms in Mother-Newborn Pairs

We found one or two similar predominant microorganisms in 12 mother-newborn pairs (85%) from among these 14 pairs in which the newborn was heavily contaminated (fig. 2). *Streptococci* were the most frequent common predominant microorganisms, occurring in 4 mother-newborn pairs. In the remaining pairs, we found different similar predominant microorganisms: *Staphylococci, Bacilli, Corynebacteria* and anaerobic cocci each in 2 pairs; *Bacteroides, Propionibacteria* and *Eubacteria*, each in one pair. In addition to that, up to 4 similar subordinate microorganisms could be detected in the investigated pairs.

Discussion

This study demonstrated that, at birth, there was a great similarity, both qualitative and quantitative, between the individual microflora of mother's vagina and their newborn's ear. The degree of colonization of the newborn significantly correlated with the count of microorganisms in its mother's vagina. In most of the investigated individual mother-newborn pairs we could reveal similar prevailing microorganisms. There were no cases of mothers and their newborns harbouring similar prevailing potentially pathogenic microorganisms.

Pertaining reports offer some conflicting results. Thus, the investigation by Graham [15] has shown that only 25% of healthy neonates harbour microorganisms on their skin immediately after birth and the latter are the same as in the vagina. Ivanov and Kulish [16] claimed that the contamination of healthy neonates with microbes varied in different clinics in a range between 7 and 30%, the most frequent contaminants being epidermic *Staphylococci*. On the other hand, Sycheva et al. [17] found microorganisms on the conjunctiva of 83% of neonates and the microbes resembled those found on their mothers' skin. It seems that the results may depend, on the

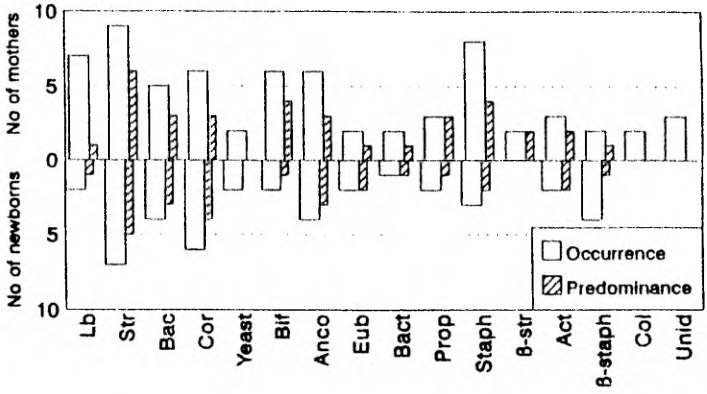

Fig. 1. Occurrence and predominance of microorganisms in vaginal samples of mothers and ear samples of newborns. Lb = *Lactobacilli*, Str = *Streptococci*, Bac = *Bacilli*, Cor = *Corynebacteria*, Bif = *Bifidobacteria*, Anco = anaerobic cocci, Eub = *Eubacteria*, Bact = *Bacteroides*, Prop = *Propionibacteria*, Staph = *Staphylococci*, β-str = β-haemolytic *Streptococci*, Act = *Actinomyces*, β-staph = β-haemolytic *Staphylococci*, Col = coliforms, Unid = unidentified bacteria.

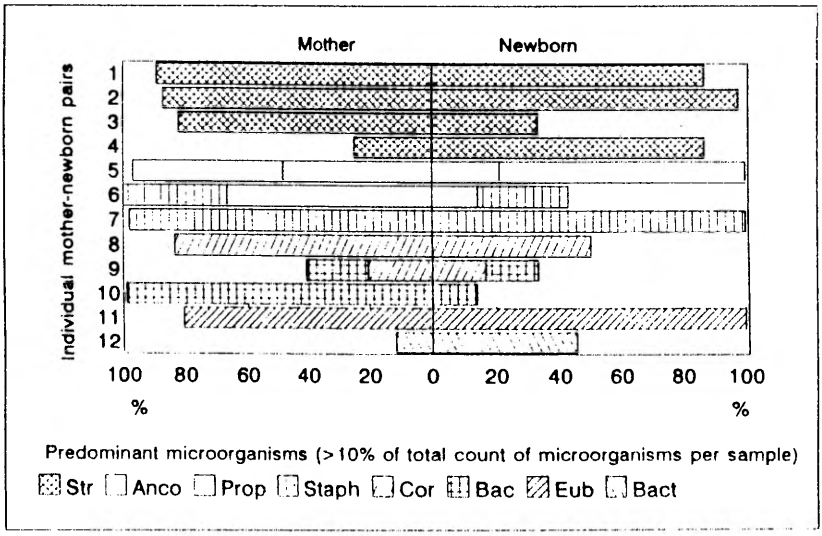

Fig. 2. Similar predominant microorganisms in 12 mother-newborn pairs. 100% = Total count of microorganisms per sample. For abbreviations, see figure 1.

one hand, on what area of the baby's body is studied and, on the other hand, on the methods of sampling and cultivation of microorganisms.

To quantify the mothers' and newborns' microflora, a special method of sampling with blood-thioglycollate-coated cotton-wool swabs was developed [10]. This method helps to avoid the loss of viability of fastidious bacteria. The material from the neonate's external ear canal was considered representative of the transfer of microorganisms from mother to the neonate, because this area seems to be rarely touched and it is thus hardly ever contaminated by the hands of the obstetrician. The predominance pattern of microflora was estimated only in the case of high density (>100) of microbes in a particular sample, to avoid registration of random distribution of microorganisms in scarcely colonized areas.

We revealed a significant correlation between mothers' vaginal microflora and their newborns' external ear canal microflora. At the same time, *Lactobacilli* frequently found in mothers were quite rarely isolated in newborns immediately after birth; however, this difference appeared to be statistically nonsignificant due to the small number of persons studied. On the one hand, this may be caused by selective attraction of neonates for adherence of *Streptococci*, as it was described by Long and Swenson [18] in oral mucosa of newborns. On the other hand, the results may depend on the relatively higher resistance of cocci to disinfectants as compared with *Lactobacilli* [19]. Consequently, the extensive vulvar cleansing during labour does not reduce the number of microorganisms, but leads to a selective transfer of maternal bacteria, unfortunately excluding *Lactobacilli*. Evidently, the idea of decontamination of the birth canal by modern antiseptics (chlorhexidine) before delivery [20] does not work properly.

We could not find any universal type of microflora or microorganisms characteristic of all mothers. All the microfloras studied were individually different both in qualitative and quantitative terms. Thus, even the previously described increase in counts of *Lactobacilli* as gestation advanced [3, 21] and similar hormonal status of women during delivery [21] could not reduce the individuality of microflora. This individuality was also common in case of the contaminated newborns and has been demonstrated while screening risk-of-infection newborns due to the contamination with opportunistic pathogens [6, 10, 22, 23]. However, all the numerous investigations have in most cases only registered either the presence or absence of potential pathogens, and their value in predicting the infectious agents is no doubt limited [24].

To overcome this drawback, we determined the predominance pattern of microorganisms in each sample, finding in most cases similar prevailing microbes in mother's vaginal and her neonate's initial microflora. It is interesting to note that we did not find in our study any mother-newborn pairs with opportunistic microorganisms (β-haemolytic *Strepto-* and *Staphylococci*, coliforms or *Candida* sp.) as prevailing microbes in both individuals.

We conclude from our study that the predominance pattern of the mother's genital microflora has significant influence on the initial microecological relations of her newborn.

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IV

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Bakteriaalne vaginoos

Reet Mändar • Tartu

bakteriaalne vaginoos, levik, etiopatogenees, diagnoos, komplikatsioonid, ravi, rasedus

Bakteriaalne vaginoos on üks günekoloogias enamuuritud tervisehäireid, kuid kliinilises praktikas diagnoositakse seda veel vähe. Intensiivsetest teadusuuringutest hoolimata on selle olemus jäänud mõneti ebaselgeks. Seetõttu esitame kirjanduse ülevaate ning seejärel oma uurimistulemused.

Mõiste ja levik. Bakteriaalne vaginoos — «ökoloogiline müsteerium» (28) — on naiste hulgas väga levinud tervisehäire, põhjustades kuni 50% tupeinfektsioonidest (7, 16). Seda sündroomi on nimetatud *Haemophilus vaginalis*'e vaginiidiks, mittespetsiifiliseks vaginiidiks, mittespetsiifiliseks vaginoosiks, anaeroobseks vaginoosiks ja amiinkolpiidiks. Praegune nimi — bakteriaalne vaginoos — on kasutusel 1984. aastast alates (13). Seda vaginiidiks nimetada ei ole õige, sest bakteriaalse vaginoosi puhul ei esine põletikulist reaktsiooni, s.t. leukotsüütide hulga suurenemist tupesisaldises.

Sümptoomid. Bakteriaalsele vaginoosile (BV) on iseloomulik: homogeenne hallikas tupevoolus; vooluse pH>4,5; voolus on iseloomuliku kalalõhnaga, mis on eriti terav, kui sellele lisada esemeklaasil mõni tilk 10%-list KOH-i. Lõhn on tingitud lenduvatest amiinidest, peamiselt putrestsiinist ja kadaveriinist); nn. võtmerakud tupesisaldises, mida on lihtne kindlaks teha, kui füsioloogilise lahusega segatud tupevoolust vaadelda 400-kordses suurenduses mikroskoobi all. Võtmerakud on tupeepiteelirakud, mis on nii tihedalt mikroobidega kaetud, et rakupiir on muutunud ebaselgeks (13).

Etioloogia ja patogenees. Bakteriaalse vaginoosi etioloogia ja patogenees on tänaseni lõplikult selgitamata. Selle sündroomi tekke eest on vastutavaks peetud selliseid mikroorganisme nagu *Gardnerella vaginalis, Mobiluncus sp., Mycoplasma hominis, Bacteroides sp.* jt. Samas on neid mikroobe leitud ka täiesti tervete naiste genitaaltraktist (4, 13, 20, 32). Praegu ollakse seisukohal, et bakteriaalne vaginoos on tupe mikroökosüsteemi tasakaalustamatus (15), mille puhul mikroaerofiilsed laktobatsillid tupes puuduvad või on neid väga vähesel hulgal, selle asemel domineerib siin kooslus eelloetletud mikroobidest (6, 21). Selline seisund kujuneb tõenäoliselt mikroobide omavaheliste suhete häire tagajärjel (20). Süsteemi tasakaalu korral toodavad mikroaerofiilsed laktobatsillid H_2O_2, mis on bakteriaalse vaginoosiga seotud mikroobide suhtes toksiline ja takistab nende ülemäärast paljunemist (9, 17). Patogeneetiliselt oluline on *Gardnerella vaginalis*'e, *Mobiluncus sp.* ja *Bacteroides sp.* võime toota suktsinaati, mis pidurdab leukotsüütide kemotaktilist aktiivsust (29). Ühtlasi on leitud, et bakteriaalse vaginoosi ja tupe normaalse mikrofloora korral esinevad *Gardnerella vaginalis*'e erinevad biotüübid (4), mis võivad olla erineva virulentsusega.

Soodustavateks faktoriteks bakteriaalse vaginoosi tekkes on peetud emakasisese spiraali olemasolu (31), suurt seksuaalpartnerite arvu (15, 31), naise hormonaalset seisundit (20). Osa autoreid eitab seksuaalse ülekande tähtsust, sest puuduvad olulised erinevused bakteriaalse vaginoosi esinemissageduses neitsite ja seksuaalselt aktiivse kontingendi vahel (5) ning mikroobide reservuaariks on pärasool (11, 12). Ka hormonaalsete mõjude suhtes ei ole ühtset seisukohta, sest bakteriaalset vaginoosi esineb peamiselt reproduktiivses eas, s.t. naissuguhormoonide kõrge nivoo foonil (20), samas aga oraalne kontratseptsioon naissuguhormoone sisaldavate preparaatidega isegi vähendab bakteriaalse vaginoosi esinemissagedust (26).

Võimalikud komplikatsioonid. Bakteriaalne vaginoos kulgeb paljudel juhtudel asümptomaatiliselt ja võib kaduda igasuguse ravita (1, 5, 31). Kuid vaginoosiga seotud mikroobid võivad astsendeeruda ning põhjustada salpingiite ja endo-

metriite (20). Samuti produtseerivad nad kantserogeenseid nitrosoamiine (25). Bakteriaalsest vaginoosist on tingitud ka mitmed raseduse ja sünnitusega seonduvad komplikatsioonid — enneaegne sünnitus, enneaegne lootekestade rebend, väike sünnikaal, korioamnioniit, sünnitusjärgne sepsis, sünnitusaelsed ja -järgsed genitaalinfektsioonid, sealhulgas endometriit (3, 15, 18, 20, 22, 24).

Diagnoos. Bakteriaalse vaginoosi diagnoos on enamasti kliiniline eespool toodud nelja kriteeriumi alusel. Et diagnoosida bakteriaalset vaginoosi, peab olema vähemalt kolm nimetatud neljast kriteeriumist (13).

Lihtne ja odav on bakterioskoopiline diagnoosimine Grami või Papanicolaou' järgi värvitud äigepreparaatide alusel (18, 30).

Bakterioloogiline diagnoosimine on kallis ja vähe informatiivne, sest bakteriaalse vaginoosiga seotud mikroobe leidub sageli ka normaalses mikroflooras ning enamik neid on kultiveeritavad ainult anaeroobsetes tingimustes. Informatiivsus suureneb, kui kasutada kvantitatiivset bakterioloogilist meetodit, see aga on keerukas ja töömahukas.

Kromatograafiameetod mikroobide ainevahetusproduktide määramiseks on meie oludes kahjuks veel vähe kättesaadav.

Ravi. Klassikaline bakteriaalse vaginoosi ravi skeem: 500 mg metronidasooli kaks korda päevas 7 päeva vältel (13). Katsetatud on ka lühemaid raviskeeme, näiteks 2 g metronidasooli ühekordselt (19), samuti muid preparaate — 200 mg ofloksatsiini kaks korda päevas. Nendega on saadud häid tulemusi (23). Ka lokaalne ravi klindamütsiinkreemiga on tulemuslik (27). Rasedatele soovitatakse happelist laktaatgeeli (2, 14) või elusaid laktobatsille sisaldavaid preparaate (10).

Uurimismaterjal ja -metoodika. Arvestades bakteriaalse vaginoosiga kaasaskäivat ohtu, jälgisime oma uurimuses Tartu Naiste-kliinikut külastavaid rasedaid. Uuriti 42 naist, igaüht 4...7 korda raseduse vältel, kokku tehti 229 uuringut.

Tupeeritise Grami järgi värvitud äigepreparaate hinnati R. P. Nugenti ja kaasautorite (24) poolt pakutud metoodika alusel, mille puhul loendatakse mikroskoobi vaateväljas suuri grampositiivseid pulkbaktereid (*Lactobacillus*'e morfotüüp), väikesi gramnegatiivseid ja gramvariaableid pulkbaktereid (*Gardnerella vaginalis*'e ja *Bacteroides*'i morfotüübid) ning kõveraid gramvariaableid pulkbaktereid (*Mobiluncus*'e morfotüüp). Vastavalt arvule hinnati iga morfotüüpi 0...4-pallisüsteemis, kusjuures laktobatsillide puhul andis suurem mikroobide hulk vaateväljas vähem palle, teiste puhul aga vastupidi. Kui kolme morfotüübi summa oli 0...3 palli, peeti tupefloorat normaalseks, kui 4...6 palli, siis vahepealseks; kui aga palle oli 7 või rohkem, siis konstateeriti bakteriaalset vaginoosi (vt. tabel). Paralleelselt hinnati preparaatides võtmerakkude esinemist.

Tabel. Mikroobide morfotüüpide jaotumus (R. P. Nugenti ja kaasautorite järgi)

Pallid	Suured gram-positiivsed pulgad	Väikesed gramnega-tiivsed ja gramvariaab-lid pulgad	Kõverad gramvariaab-lid pulgad
0	4+	0	0
1	3+	1+	1+ või 2+
2	2+	2+	3+ või 4+
3	1+	3+	
4	0	4+	

0 — morfotüüp puudub
1+ — <1 vastava morfotüübi mikroskoobi vaateväljas
2+ — 1...4 morfotüüpi vaateväljas
3+ — 5...30 morfotüüpi vaateväljas
4+ — >30 morfotüübi vaateväljas

Uurimistulemused ja arutelu. Bakteriaalset vaginoosi leiti 31%-l 229 uuringust. Vähemalt üks vaginoosi episood raseduse vältel esines 20 naisel, neist 14-l rohkem kui pooltes uuringutes, sealhulgas 7 naisel leiti vaginoos kõigis raseduse ajal tehtud uuringutes. Kordagi ei leitud seda 22 naisel.

Kõigist uuringutest olid pooled vahepealse tupefloora poegeldajaiks ning tupefloora oli normaalne ainult 19%-l uuringutest. Ei olnud ühtki naist, kelle mikroflora oleks kogu raseduse ajal normaalne olnud (0...3 palli).

Võtmerakke leidus 68-s 72-st bakte-

riaalse vaginoosiga preparaadist. Neid leiti ka kahel juhul 114 vahepealse, mitte kordagi aga normaalse mikrofloora korral. Seega meie uuring kinnitab võtmerakkude head korrelatsiooni bakteriaalse vaginoosiga (r=0,93).

Meie poolt uuritud naistel võis bakteriaalset vaginoosi täheldada küllaltki sageli. Kirjanduse andmeil on seda täheldatud 10...26%-l rasedaist (8, 18, 22, 31). Huvitav on ka asjaolu, et ühtede puhul on vaginoos ebapüsiv, teiste puhul aga esineb seda stabiilselt kogu raseduse ajal. Raseduse lõpupoole võib märgata vaginoosi mõningast vähenemistendentsi, eriti viimases uuringus enne sünnitust.

Arvestades bakteriaalse vaginoosiga kaasnevat ohtu, peaks selle korduv leid tegema arstid valvsaks ning selliseid patsiente tuleks ravima hakata.

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Summary

Bacterial vaginosis. Bacterial vaginosis, the dysbalance of vaginal microbial ecosystem, is a very common disease 42 pregnant women were investigated, each 4...7 times during pregnancy. Gram-stained smears of the vaginal discharge were examined for different microbial morphotypes and for "clue cells". 20 women out of 42 had at least one BV episode during pregnancy. 7 of them had stable BV in all samples. "Clue cells" have a good correlation with BV (r=0,93). The etiopathogenesis, complications, diagnostics and treatment possibilities of BV are also discussed.

Üld- ja Molekulaarpatoloogia Instituut

V

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9

Development of Individual Lactic Acid Microflora in the Human Microbial Ecosystem

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I. GENERAL INTRODUCTION

The very first days of our existence pass pleasantly in the deep warm safety of our mother's womb, and it must be a shock of supreme power for a neonate to enter this world with its cosmic numbers of microbes. However, "wise Nature" could not have planned the first contamination of a neonate to happen at random. It is the task of biological sciences to find out the natural precautions. One can suggest it to be the normal microflora, inhabiting the genital tract of a healthy mother, on which the newborn's defense mechanism relies. The predominant microorganisms of the vagina are microaerophilic and anaerobic lactobacilli, streptococci and some other lactic acid producing microbes (Larsen and Galask, 1980; Cook et al., 1984;).

Lactobacilli, one of the most frequent Gram-positive bacteria in the human microflora, usually inhabit various organs as innocuous commensals. Since the turn of the century human lactobacilli have been considered contributors to human health (Metschnikoff, 1908). The interest in their existence in different areas of the body and their role in the host and microflora-related physiochemical conditions in the organism has become one of the subjects of a comparatively new research field—microbial ecology (Haenel, 1957a,b, 1980;

Reuter, 1965, 1975; Dubos, 1966; Gilliland et al., 1975; Goldin and Gorbach, 1984; Gorbach, 1990).

In this chapter an attempt is made to summarize and interpret the current state of our knowledge of the composition, maintenance and formation of human individual lactoflora.

The recognition of lactobacilli as an important part of the human microbial ecosystem and the understanding of various interconnected influences of that system is our starting point. Several researchers have underscored (Reuter, 1965; Lencner, 1973, 1984, 1987; Mitsuoka et al., 1975) the large individual differences of lactoflora, expressed in numbers and species' composition. Population studies and clinical investigations are the main sources of information about the effect of the genetic background of hosts, and their age and health on the persistence of lactobacilli in different persons (Lencner, 1973; Mikelsaar et al., 1982, 1984; Hanson et al., 1989, 1990). The data of the transmission of lactobacilli from mother to neonate during the birth, mode of delivery, hospital conditions, and the effect of feeding upon the establishment of lactoflora in economically differently developed countries contribute to the understanding of the influence of various environmental factors for neonatal ecology of lactic acid bacteria (Raibaud et al., 1980; Bennet, 1987; Hall et al., 1990).

The factors determining the development of individual lactic acid microflora are not completely known yet, and the question about which strains become residential in various human organs remains an object of discussion (Tannock et al., 1990b). The in vitro studies determining the adherence capacities of lactobacilli to eukaryotic cells and other microorganisms are useful for understanding the selective adherence of lactobacilli to a particular host (Fuller, 1975; Brilis, 1983; Dalin and Fisch, 1985; Wadström and Aleljung, 1989). The relationship between the colonizing properties and the in vitro determined antimicrobial substances of particular strains of lactobacilli has not been solved yet.

Direct assertion of the mechanisms by which lactobacilli colonize various areas of a human body require advanced experimental techniques (Savage, 1977, 1989). Here, the selection of any appropriate animal model and particular bacterial strains may be crucial. One point of importance seems to be that the lactobacilli should originate from the animal species studied (Tannock, 1983).

The practical goal of the investigations of lactoflora formation is to develop anti-infectious treatments such as controlled colonization of neonates with biologically highly active strains of lactobacilli (Perdigon et al., 1986; Goldmann, 1988; Moshchich et al., 1989). The revised theoretical basis of formation of lactoflora may improve the development of the infants so treated.

C. Stability of Human Microflora

1. *Individuality of Microflora*

The individual differences in the species composition and the numbers of microorganisms of the skin (Lynch and Poole, 1979; Kearney et al., 1984; Leeming et al., 1984), vagina (Reuter, 1965, 1975; Lencner, 1973; Solovyeva, 1986, 1987; Kasesalu et al., 1990), and intestine (Holdeman et al., 1976; Mitsuoka and Ohno, 1977; Finegold et al., 1983) are documented by many investigators, who have studied the microbial ecosystems of adult organisms. Controversial data have been obtained about the stability of individual microflora of various biotopes.

The numbers of microbes in the fecal microflora of a person vary greatly for period of some months (Gorbach et al., 1967) or even a few days (Meijer-Severs, 1986). However, Holdeman et al. (1976) found the quantitative composition of fecal microflora to be very specific for a particular host. The stability of the quantitative composition of the fecal microflora of a particular host means mostly the persistence of stable quantitative relations between the most frequent and predominant groups of microorganisms (Zubrzycki and Spaulding, 1962; Mitsuoka and Ohno, 1977). Thus, after a whole year's study of the fecal microflora of 10 healthy volunteers, stable relationships between the numbers of different aerobic and anaerobic groups of microorganisms were ascertained (Mikelsaar, 1969).

Only few species of microorganisms inhabiting the GI have been followed for their persistence in the same person during long periods. The stable occurrence of the same biotypes of bifidobacteria (Gossling and Slack, 1974), bacteroides (Johnson, 1980; Moore et al., 1979) and certain phenotypes of *E. coli* (Kuhn et al., 1986) has been proved. The latter microbes are suggested to be especially adapted for colonizing the human intestine. It is obvious that the host- and microflora-derived physiochemical conditions of microbial biotopes cannot be too similar for different persons and in that sense microbial ecosystems are always deeply individual, having specific interindividual peculiarities (Haenel and Bendig, 1975).

The microbial ecosystem as a whole is successfully characterized by biochemical studies determining the metabolites of the microorganisms excreted from the human body. The very specific and personally stable composition of various bacterial metabolites excreted by urine or feces has been revealed by several researchers (Hoverstad et al., 1984; Weaver et al., 1989; Midtvedt, 1989a,b, 1990; Siigur et al., 1991). These data also confirm the occurrence of individually specific microflora in humans.

2. *Individual Stability of Lactoflora*

a. Feces. The number of lactobacilli in fecal samples of different persons appears to be quite different (Mitsuoka and Ohno, 1977; Knoke and Bernhardt, 1985). In a survey over a long period (Mikelsaar, 1969; Mikelsaar et al., 1982, 1984) of the number of lactobacilli in the faeces of 10 healthy volunteers quite stable and characteristic quantities were revealed (Table 2). The range of the number of lactobacilli in the feces of four persons (nos. 1, 7, 9, 10) was low. Three of them maintained this situation during the study, although they grew older, had several failures of their health, and used some medicines. The variation of different estimations of the number of lactobacilli of the investigated persons (except no. 7) did not significantly exceed (<1 log cfu/g, the normal error of quantification) the first examination range. It is important to mention that all 10 volunteers are sill in full health even now, when more than 25 years have passed.

Table 2 Quantity of Lactobacilli in the Feces of Healthy Persons During 15 Years Follow-Up

	Quantity of lactobacilli (log cfu/g)		
	I investigation[a]	II investigation	
No. of persons	Range of 6–8 samples		III investigation
1	5.2–7.0	5.6–6.0	5.9
2	5.9–7.5	6.3–7.5	6.0
3	5.8–7.4	6.8–7.5	7.8
4	5.7–7.7	6.9–7.3	7.6
5	5.7–7.7	7.9–8.3	7.3
6	6.2–7.6		7.0
7	5.5–6.7	6.7–8.3	7.3
8	6.3–8.1	6.6	7.3
9	5.5–6.7		7.1
10	6.2–7.0	7.0	

[a]At the I investigation the age of patients was 23–44, at the II, 30–51, and at III, 38–57 years.

In addition to the stability of the number of lactobacilli in feces, the stable persistence of their fecal lactobacillar species (Table 3) was revealed (Mikelsaar et al., 1975, 1982). Among two to four species of lactobacilli isolated from every adult volunteer, one or two species occurred repeatedly and in four persons we could even observe the persistence of the same biotype. Similarly, in a study of the seasonal variation of fecal lactoflora, the investigation of feces of children aged 7–12 revealed that the same biotypes of lactobacilli were detected in 8 out of 11 children (Golyanova, 1972).

The prolonged biological isolation of healthy persons during special training or space flight periods of astronauts caused some shift to opportunistic microorganisms (Shilov et al., 1972). Yet the close physical contact could not eliminate the individual specificity of their lactoflora in terms of species or bacterial number (Lencner et al., 1973, 1981, 1984).

b. Vagina. The number and composition of vaginal lactobacilli are significantly influenced by the hormonal status of the host (Levin, 1968; Brilene and Brilis, 1986; Redondo-Lopez, 1990). The flat epithelium of the vagina contains much glycogen, which is more readily converted into lactic acid during pregnancy, thus creating a more beneficial environment for colonization by lactobacilli, nonhemolytic streptococci, and other lactic acid bacteria (Larsen and Galask, 1980).

Unfortunately, few studies of the vaginal microflora of pregnant women have included a control group of nonpregnant women (Redondo-Lopez, 1990; Tashjian et al., 1976). In that sense our unpublished data (Mändar et al.) comparing the lactoflora of healthy pregnant women with those with a threatened abortion seem to be very informative. It was demonstrated that the relative amount of lactobacilli in 24 women out of 42 increased as gestation advanced (Fig. 2). This tendency started in women with a threatened abortion somewhat earlier (in 24–26 weeks of pregnancy) than in the control group (in 32–34 weeks). It could be explained by the influence of hormonal preparations administered to support the gravidity in cases of threatened abortion. The predominance of lactobacilli may be considered a preventive mechanism offering protection to the fetus and neonate just before labor. After delivery, such a bacterial status disappears (Bartlett et al., 1977; Stahl and Hill, 1986).

The problem of individual stability of human microflora is closely connected to its formation, mainly during vaginal delivery. However, the factors determining which bacterial strains colonize and persist in the infant from the very first days of its life are not known yet (Tannock et al., 1990b). The main reason for that may be the complexity of interactions in a human's microbial ecosystem.

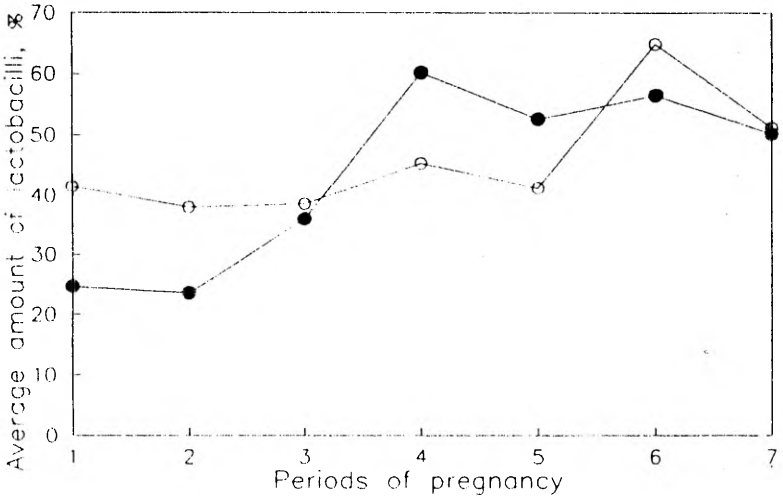

Figure 2 Average relative amounts of lactobacilli in vaginal microflora in women harboring them. 1–10 weeks of gestation age; 2—11–16 w; 3—17–22 w; 4—24–26 w; 5—28–30 w; 6—32–34 w; 7—36–38 w. o———o control group; • – – – • women with threatened abortion.

III. ESTABLISHMENT OF NEONATAL MICROFLORA

The pathways, manners, and various factors causing the contamination of the sterile fetus during labor and of the newborn in the first hours and days of its life and also determining its microflora formation, have been drawing scientists' attention for nearly 100 years (Tissier, 1905; Cooperstock and Zedo, 1983; Goncharova et al., 1987; Hall et al., 1990).

In the formation of normal human microflora two stages can be distinguished: the acquisition of microorganisms by contamination with mother's microorganisms or with other environmental microbes and the successive colonization of different habitats of the neonate (Savage, 1977).

The real colonization means the persistence of microorganisms in the biotope even up to 14 days after their first appearance (Van der Waaij, 1973, 1988).

A. Transmission of Microorganisms from Mother to Infant

Several authors suggest that the neonate is sterile during the period of intrauterine life (Haenel and Bendig, 1975; Davies and Grothefors, 1984). Contamination with commensal bacteria, derived from the microflora of mother's vagina, intestine, and skin and from the environment occurs soon after birth (Bullen, 1977; Ross and Needham, 1980; Rotimi and Duerden, 1981; Bennet and Nord, 1987; Ekwempu et al., 1982; Enhtuja, 1984). Many of these microbes are unable to colonize habitats in the neonate and disappear soon after birth, whereas other microorganisms remain or may support the successive colonization during the early life period to form climax communities in the adult (Savage, 1977, 1989).

1. Infectious Microorganisms

The interest of investigators in the above field has focused on two directions. The widest trend of investigations has been prompted by the practical goal of preventing ante- and perinatally derived infections, such as listeriosis, toxoplasmosis, chlamydial, and various viral infections (Iwasaka et al., 1986; Maciejewski et al., 1987). These infections are generated by pathogenic microorganisms the transmission of which from mother to her neonate has been documented (Davies and Gothefors, 1984).

In the last years, no less attention has been paid to the transmission of the potentially pathogenic (opportunistic) microorganisms like *Staphylococcus aureus, Clostridium difficile*, and *Group B Streptococci* (GBS) from the mother to her neonate. These bacteria may or may not cause various early postnatal infections (Manso et al., 1986; Rudigoz, 1988; Tullus et al., 1989; Kay et al., 1990).

Lately, it has been suggested that they have a certain role even in preterm labor connected with extra- and intra-amniotic infections (Iams et al., 1987; McGregor et al., 1988, 1990).

It has become known that these opportunistic microorganisms are kept in mutual or commensal interactions with the host by various factors characteristic to the macroorganism and other microorganisms of the indigenous microflora (Hungate, 1984; Savage, 1989). It has been speculated that the resident strains of the indigenous microflora, through various mechanisms, protect the baby from the very beginning of its life from the randomly acquired opportunistic strains (Haenel and Bendig, 1975; Davies and Gothefors, 1984).

Thus the second direction of the investigations of microflora transmission from mothers to their newborns is concentrated on the problem of how the colonization controlling indigenous microflora is formed. In this respect there has been a resurgence of interest towards the colonization of the neonate by lactobacilli (Hall et al., 1990; Tannock et al., 1990b).

2. *Indigenous Microflora*

Several studies have described the early skin contamination of the newborn, comparing the neonate's and its mother's microfloras. The investigation by Graham (1975) has shown that only 25% of healthy neonates harbor microorganisms on their skin immediately after birth and the latter are similar to those of the vagina. Ivanov (1985) has shown that the contamination of healthy neonates with microbes varies in different clinics from 7% to 30%, the most frequent contaminants being epidermic staphylococci.

It may be possible that the results of the investigations depend on the location in the baby from which the material was taken. So Bakuleva et al. (1984) claim that the gastric aspirates of neonates were bacterioscopically and bacteriologically uncontaminated. Deshchetkina et al. (1990) described sterile samples of meconium in 83% of the investigated newborns. On the other hand, Sycheva et al. (1986) found microorganisms on the conjunctiva of 83% of the neonates and the microbes resembled those detected on mother's skin. However, the conflicting results may also depend on the different media and methods used for the search of various groups of microorganisms. Very important data are gained when comparing, on the one hand, the mother's vaginal microflora with that of the neonate after vaginal delivery or, on the other hand, mother's amniotic fluid with the newborn's microflora after a caesarean section.

Cesarean section thoroughly alters the colonization patterns in newborn infants. Anaerobic colonization is delayed and there appears an overgrowth by enterobacteria (Rotimi et al., 1985). The colonization of newborns delivered by cesarean section occurs during the first days of life by bacteria provided by the

outer environment (Neut et al., 1987). While Lennox-King et al. (1976) find the most common sources for *Escherichia coli* colonization to be the nurses' hands and the contaminated air, Bezirtzoglou and Romond (1990) deny the role of hospital environment and the type of feeding in Enterobacteriaceae colonization.

Recently Torres-Alipi et al. (1990) could reveal no correlation between the microorganisms isolated from the amniotic fluid and the neonate's oral cavity after a cesarean section. In case of the neonates obtained by vaginal delivery there was, however, a correlation between the microorganisms of the oral cavity and the maternal vaginal flora.

Rotimi and Duerden (1981) in their now classical study found that immediately after birth in 52% of cases the lactobacilli were present both in childrens' mouths and in mothers' vaginas. Only in rare cases lactobacilli were isolated either from the mother (14%) or from her neonate (9%).

We compared the microflora of 21 healthy mothers during delivery with that of their newborns also immediately after birth. We examined the skin microflora of mother's perineum and her baby's external ear canal where the amniotic fluid may possibly persist and which remains untouched by the hands of the medical personnel. Aerobic and anaerobic microorganisms were present both in perineal and ear samples (Mikelsaar et al., 1989). In mothers and in their neonates 13 and 11 groups of microorganisms were detected, respectively (Figs. 5, 6). The most frequent microorganisms were the same both in mothers and their newborns: lactobacilli, epidermic staphylococci, and nonhemolytic streptococci. Various combinations of microbes were found mostly dissimilar in the investigated mothers. The maternal perineum was heavily colonized with microorganisms in 75% and the infants' ear skin in 24% of the cases (more than 100 cfu/tampon) and there was no real accordance in the quantitative characteristics of microbial densities between mothers and their infants. Yet, if we studied, using the same methods, the maternal vaginal and neonatal ear microfloras (19 mother–newborn pairs) we could see a close correlation between the colonization rates of the above-mentioned biotopes ($p < .01$; Kasesalu et al., 1991). We found similar predominant groups of microbes in 12 pairs out of 19 (63%). Of the remaining seven pairs, in four babies we could not detect any of the microorganisms searched for. Thus, it has been demonstrated that maternal vaginal microflora species and its quantitative characteristics directly influence the initial contamination of the newborn.

The assumption that the source of the bacteria which finally colonize the infant is the maternal vagina, has not been completely proved yet. Lately discrimination tests permitting the comparison of bacterial strains isolated from maternal and infant sources have been developed. Plasmid profiling has proved a useful technique for that (Davies et al., 1981; Farrar, 1983).

Tannock et al. (1990b) compared the plasmid profiles of the microbes of the family Enterobacteriaceae, lactobacilli and bifidobacteria cultured from the vaginal, oral, and rectal swabs of birth-giving mothers with the strains detected in the feces of their infants 10 and 30 days after birth. Lactobacilli inhabiting the mothers' vaginas did not appear to colonize the infants' digestive tracts, but the authors got evidence of the transmission of fecal isolates of enterobacteria and bifidobacteria from mothers to their infants.

VI

In Manuscript

Susceptibility Patterns of Vaginal Lactobacilli Isolated from Pregnant Women in Estonia and USA

Reet Mändar, Mall Türi, Stephen D. Allen and Marika Mikelsaar

SUMMARY

During pregnancy, the vaginal lactobacilli have been considered to be important for the health of woman and her newborn. Hence, it is advantageous to avoid the use of antibiotics that are highly active against lactobacilli.

Our aim was to study the susceptibility to antibiotics of vaginal lactobacilli in pregnant women in Estonia and USA.

36 strain of lactobacilli were obtained from 13 consecutive pregnant women at Tartu Maternity Hospital (Estonia) and from 17 consecutive pregnant women at Indiana University Hospital (Indiana, USA). The isolated strains of lactobacilli were tested for their susceptibility to 15 antibacterial agents using Kirby-Bauer disc diffusion method.

All investigated strains of lactobacilli were susceptible to ampicillin, amoxicillin and imipenem. Single strains were resistant to penicillin, cefotaxime, erythromycin and doxycycline. Some 13-17% of strains were resistant to cefazoline, ceftazidime and vancomycin, nearly a quarter to clindamycin, about half of strains were resistant to gentamicin and cefoxitin. We could observe high numbers of strains resistant to ofloxacin and aztreonam among lactobacilli investigated by us. The lactobacilli of Estonia and USA showed different susceptibility patterns only in case of ceftazidime (5.3% of resistant strains in Estonia and 29.4% in USA, $p=0.05$) and cefoxitin (87.5% and 23.5%, respectively, $p=0.0003$).

We could observe that vaginal lactobacilli are not uniform as to their susceptibility to antibiotics. It should be important for clinicians to know that treatment with aztreonam and ofloxacin seems to be safer for vaginal lactoflora in both countries.

INTRODUCTION

Lactobacillus spp. make up a major part of the normal vaginal microflora in women of reproductive age. A number of studies suggest that lactobacilli have a protective effect on the body maintaining the ecological balance of the vaginal flora [1,2]. During pregnancy, the number of vaginal lactobacilli usually increases which

has been considered to be important for the health of woman and her newborn [3]. In some situations, like urinary tract infections, a pregnant woman unavoidably has to be treated with antimicrobial drugs [4,5]. In such cases, beside the other contraindications, it is advantageous to avoid the use of antibiotics that are highly active against lactobacilli. However, as in the literature there is little information concerning the susceptibility of lactobacilli to antibiotics, it is difficult to follow this suggestion [6,7].

At the same time, the increasing resistance of microorganisms which probably is the result of the extensive use of antibiotics has become a worldwide problem [8,9]. Some antibiotics, like third-generation cephalosporins and quinolones have been in wide use for a comparatively shorter period in Estonia (one of the Baltic countries) than in USA [10,11]. Thus, it can be supposed that microorganisms in Estonia and USA may harbour different susceptibility patterns.

Our aim was to study the susceptibility to antibiotics of vaginal lactobacilli in pregnant women in Estonia and USA

MATERIAL AND METHODS

Specimens

Lactobacilli were obtained from 13 consecutive pregnant women (Group A) at Tartu Maternity Hospital (Estonia) and from 17 consecutive pregnant women (Group B) at Indiana University Hospital (Indiana, USA). High vaginal swabs were taken at their regular control during pregnancy.

Isolation

Nineteen strains were isolated from Group A and 17 strains from Group B, altogether 36 strains of lactobacilli. For isolation of lactobacilli three different media were used: MRS-agar (MERCK) for Estonian women, Anaerobic Blood Agar (BBL) and Tomato juice agar [12] for USA women. The plates were incubated for 2-3 days at 37°C in an environment with 10% of CO_2 (Group A) or in 85% N_2 + 5% CO_2 + 10% H_2-environment (Group B). Lactobacilli were identified by colonial and cellular morphology and negative catalase production. Our aim was not to make a statistically valid survey of what species were obtained from different samples, but merely to obtain a pool of vaginal lactobacilli.

Susceptibility testing

The isolated strains of lactobacilli were tested for their susceptibility to 15 antibacterial agents (Table 1) using Kirby-Bauer disc diffusion method [13].

Lactobacilli were suspended in thioglycollate broth to the density of 0.5 McFarland standard. The suspensions were inoculated onto agar plates using swabs and the BBL Sensi-Disc Susceptibility Test Discs were added. The plates were incubated at 37°C for 48 h. For Group A, we used Fastidious Anaerobe Agar with 5% of human blood (F.A.A., LAB M) in the environment of H_2 + 4-10% CO_2, for Group B, we used Blood Agar Anaerobe (Carr Scaborough Microbiologicals Inc.) in the environment of 85% N_2 + 5% CO_2 + 10% H_2. As the breakpoint for every antibiotic was considered the growth inhibition zone diameter between "resistant" and "intermediate" according to the manufacturer's instructions, conformed to the criteria of the National Committee for Clinical Laboratory Standards [14].

Statistical methods

The data were analyzed using Wilcoxon rank test and correlation analyse using program "Statgraphics".

RESULTS

Susceptibility pattern of vaginal lactobacilli

The data of susceptibility/resistance pattern of vaginal lactobacilli of all investigated pregnant women against fifteen antibiotics are presented in Table 1. All investigated strains of lactobacilli were susceptible to ampicillin, amoxicillin and imipenem. Single strains were resistant to penicillin, cefotaxime, erythromycin and doxycycline. Some 13-17% of strains were resistant to cefazoline, ceftazidime and vancomycin, nearly a quarter to clindamycin, about half of strains were resistant to gentamicin and cefoxitin. We could observe high numbers of strains resistant to ofloxacin and aztreonam among lactobacilli investigated by us. Vancomycin-resistant strains of lactobacilli tended to be ofloxacin-susceptible, and *vice versa* ($r=-0.81$, $p<0.05$).

Comparison of lactobacilli isolated from Estonian and USA women

The lactobacilli of Groups A and B showed different susceptibility patterns only in case of ceftazidime (5.3% of resistant strains in Group A and 29.4% in Group B, $p=0.05$) and cefoxitin (87.5% and 23.5%, respectively, $p=0.0003$). In case of cefazoline (5.3% and 23.5%) and vancomycin (5.3% and 23.5%) the difference was statistically not significant (Fig 1). Additionally we tested our Estonian strains of lactobacilli which showed resistance to ofloxacin against ciprofloxacin and found the same resistance pattern.

Table 1
Susceptibility of vaginal lactobacilli (n=36) to antibiotics

Antibiotics	Percentage of isolated strains of lactobacilli (n=36)	
	susceptible	resistant
Penicillins		
Penicillin	97.2	2.8
Ampicillin	100	0
Amoxicillin-clavulanate	100	0
Cephalosporins		
Cefazolin	86.1	13.9
Cefotaxime	97.2	2.8
Ceftazidime	83.3	16.7
Cefoxitin	45.5	54.5
Other beta-lactams		
Aztreonam	8.3	91.7
Imipenem	100	0
Aminoglycosides		
Gentamicin	60.6	39.4
Quinolones		
Ofloxacin	20.6	79.4
Tetracyclines		
Doxycycline	97.0	3.0
Macrolides		
Erythromycin	97.2	2.8
Lincosamides		
Clindamycin	72.7	27.3
Glycopeptides		
Vancomycin	86.1	13.9

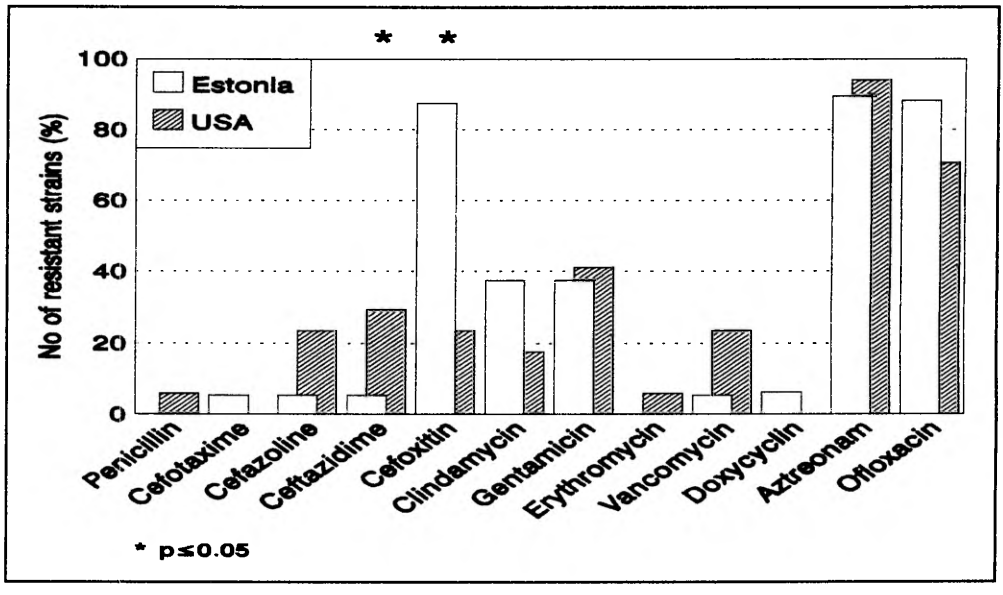

Fig. 1. Number of resistant strains among vaginal lactobacilli isolated from Estonian ans USA women

DISCUSSION

We could observe that vaginal lactobacilli are not uniform as to their susceptibility to antibiotics. Also Hamilton et al. [6] have found that lactobacilli are heterogenous in terms of tolerance to antibiotics and so it is difficult to predict their sensitivity pattern.

Our results correspond to data of a number of previous studies showing that most strains (up to 80%) of lactobacilli are susceptible to penicillins (penicillin G, ampicillin, amoxicillin-clavulanate), also to other beta-lactam agents such as imipenem [6,12,15].

Susceptibility of lactobacilli to cephalosporins seems to be curious. The high numbers of lactobacilli resistant to such expanded-spectrum cephalosporins as cefoxitin showed susceptibility to cefazolin, cefotaxime and ceftazidime. The susceptibility of some few lactobacilli strains to aztreonam is also hard to understand. Usually aztreonam binds primarily to penicillin binding protein (PBP) 3 of gram-negative bacteria and it is not thought to be active against gram-positive bacteria and anaerobes. The possible explanation might be that the inhibition of peptidoglycan synthesis was not the only mechanism of action in lactobacilli but also some other mechanisms of different efficacy might play a certain role, like

triggering of membrane-associated autolytic enzymes or inhibition of bacterial endopeptidase and glycosidase [16].

As concerns quinolones, we have found that most of the strains are resistant to ofloxacin. At the same time, there are controversial data in the literature about the newer quinolones: some 52...100% of strains have been claimed to be susceptible to ciprofloxacin [6,12]. According to our data, the tested Estonian vaginal lactobacilli occurred to be similarly resistant both to ofloxacin and ciprofloxacin.

The majority of strains of lactobacilli isolated by us were susceptible to erythromycin and clindamycin termed against gram-positive bacteria and doxycycline defined as broad-spectrum antibiotic (16).

Different investigators have found some strains of lactobacilli to be resistant to vancomycin [7,15,17]. This was revealed also by our study, where nearly 14% of strains were found to be resistant to vancomycin. The vancomycin resistance has been included into identification schemes of lactobacilli [15,17]. In our previous study we found that susceptibility to vancomycin can be determined by species of lactobacilli: *L. helveticus, L. delbrueckii ssp. lactis* and *L. acidophilus* were susceptible to vancomycin, but *L. delbrueckii ssp. delbrueckii, L. salivarius* and all strains of facultatively and obligately heterofermentative lactobacilli showed resistance to it [18]. At the same time it is important to mention that vaginal flora often consists of different species of lactobacilli. The most frequent ones seem to be *L. acidophilus, L. jensenii, L. casei, L. plantarum* and *L. brevis* [1,19], hence, belonging to both susceptibility groups. Similarly the finding that quinolones-resistance was negatively correlated with vancomycin-resistance proves the idea that susceptibility of lactobacilli to some antibiotics is mainly species-determined.

We could note some differences between the two groups of pregnant women studied in both countries. The strains of lactobacilli in Estonian women appeared to be more susceptible to ceftazidime, but more resistant to cefoxitin. In Estonia both cefoxitin and ceftazidime have been in hospital use mainly since 1992 [10,11]. Thus, the longer experience in cephalosporins-treatment in USA and the undiscriminated use of antimicrobial drugs predicted to Estonia as a postsocialist country do not seem to be the reason for different susceptibility patterns of vaginal lactobacilli in both countries. More likely this may be associated with the individual differences in human microflora species composition [3].

As concerns the choice of antibacterial drugs for pregnant women in case of urinary tract infections the suggested ampicillin and cephotaxime do not seem to be the best as unavoidably damaging the vaginal lactobacilli. It should be important for clinicians to know that treatment with aztreonam and ofloxacin seems to be safer for vaginal lactoflora in both countries.

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