Lactobacillus iners
and the normal vaginal flora

Tell Jakobsson
MD

Clinical Microbiology
Department of Clinical and Experimental Medicine
Faculty of Health Sciences
Linköping University
Sweden

Linköping 2008
Abstract

The ecological niche of the vagina contains a large number of different microbes that are constantly interacting with each other and the host. Culture methods have not been sufficient in order to resolve the complexity of the normal vaginal flora. Further, the methods for delineating normal flora from not normal flora are not easily handled and are traditionally not based on culture but on microscopy of elements of the vaginal fluid. In the work presented in this thesis, an international collaboration was established that pin-pointed some of the difficulties in classifying vaginal floras, including staining, sampling, and discordance when lactobacilli are few in number, and that emphasized the importance of the size of the vision field in microscopes. As lactobacilli are prominent members of the normal vaginal flora they need to be carefully classified if further work towards more robust scoring tools is to be achieved.

Phenotypic methods have not been able to separate the closely related \emph{Lactobacillus} species of the vagina. Progress in molecular biology has provided possibilities to characterize these lactobacilli, which are mainly from the \emph{Lactobacillus acidophilus} group. In this work a large number of strains collected by true random sampling were subjected to RAPD-PCR, TTGE and multiplex PCR for species identification. The major species found were \emph{L. crispatus}, \emph{L. gasseri} and \emph{L. jensenii} and the recently described \emph{L. iners}. The presence of \emph{L. iners} has not been detected in previous studies due to its special nutrient requirements. Development of pyrosequencing technology also made it possible to match signatures of the two variable regions V1 and V3 of the 16S rRNA gene of the vaginal lactobacilli and identify them to the species level in a high throughput manner. The study confirmed that the dominating flora in women with normal vaginal flora comprises the four species mentioned previously.

Repetitive sampling during IVF-treatment with highly varying oestrogen levels demonstrates changes that possibly occur during changes in the natural life cycle. Furthermore, \emph{L. iners} was found to be the first species to be established after spontaneously resolved or treated Bacterial Vaginosis.

These findings can be of help in developing new strategies for regaining and retaining the normal vaginal flora.
List of papers

This dissertation is based on the following papers:


Related publications by the author

VI. Larsson PG, Fähräeus L, Carlsson B, Jakobsson T, Forsum U; Premature study group of the Southeast Health Care Region of Sweden. Late miscarriage and preterm birth after treatment with clindamycin: a randomised consent design study according to Zelen. BJOG. 2006;113:629-37.


### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BV</td>
<td>Bacterial vaginosis</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>IL1ra</td>
<td>Interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>IVF</td>
<td>In vitro fertilization</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridisation</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannose-binding lecithin</td>
</tr>
<tr>
<td>PAP smear</td>
<td>Papanicolaou smear</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RAPD</td>
<td>Randomly amplified polymorphic DNA</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TTGE</td>
<td>Temporal temperature gradient gel electrophoresis</td>
</tr>
</tbody>
</table>
Table of contents

Abstract 3
List of papers 5
Abbreviations 6
Table of contents 7
INTRODUCTION 9
Vaginal lactobacilli 10
Bacterial Vaginosis 14
Diagnosis of Bacterial Vaginosis 15
MATERIAL AND METHODS 18
Microbiology in vaginal secretions as observed in the microscope (I) 18
  Collection of samples
    Wet smear composite clinical criteria of Amsel
    Nugent’s scoring system of Gram-stained smears
    Statistics
Characterization of the dominating flora in healthy women with
  normal vaginal flora (II and III) 20
    Study population
    Collection of samples
    Nucleic acid based techniques
      Randomly Amplified Polymorphic DNA-PCR
      Temporal Temperature Gradient gel Electrophoresis
      Multiplex PCR
16S rDNA sequencing

Pyrosequencing

Characterization of the dominating flora in women during IVF treatment (IV and V) 24

Study population
Hormonal assessment
Collection of samples

Bioinformatics

RESULTS AND DISCUSSION 26

Microbiology in vaginal secretions as observed in the microscope 26
Dominating normal vaginal flora in healthy Swedish women. 30
Dominating normal vaginal flora in patients during IVF treatment. 32

GENERAL SUMMARY 35

ACKNOWLEDGEMENTS 36

REFERENCES 39

Papers I-V 47
INTRODUCTION

The orifices of the human body are covered by mucous membranes that are physiological barriers to intrusion from foreign organisms, chemicals and other objects. To ensure proper protection it is generally assumed that an intact bacterial flora is required. One example of this is the vaginal bacterial flora that constitutes a normal part of female physiology from foetal life until death. The flora changes in a typical manner during the female life cycle. At birth, the vagina is sterile. After only a few days, when oestrogen from the mother has led to an increase of the glycogen content in the vaginal epithelial cells, colonization by lactobacilli from the mother occurs concomitantly(38). With oestrogen levels slowly diminishing, glycogen disappears, and thereby the prerequisite for the dominance of the lactobacilli(63). During childhood, skin commensals and bowel bacteria colonize and dominate the microbial content of the vagina. At the time of menarche, the rise in oestrogen increases glycogen deposition in the vaginal epithelial cells, which is a prerequisite for the development of the adult vaginal microflora. This flora is predominant until menopause, when it is replaced with a flora similar to the flora found prior to the menarche, unless hormonal replacement therapy is started(10).

The facts related above are part of the medical knowledge acquired over the years, but the finer details of the composition and role of the vaginal flora are still a matter of debate. This is mainly due to the fact that studies of microbial ecology in the vagina have been hampered by a lack of discriminating tools for the study of the flora itself, as well as not completely resolved questions relating to the categorization of physiological and abnormal states of the flora as normal healthy flora, bacterial vaginosis, other abnormal flora types, etc.(16). In the
present studies more detailed descriptions are given of the kinds of lactobacilli that form part of the normal flora in healthy women of childbearing age. The studies also seek to define how the dominant lactobacilli flora changes over time in women undergoing IVF-treatment in order to further define the co-variability of the flora and oestrogen levels.

Vaginal lactobacilli

The first microbiological study of the female vagina was mainly descriptive(18), and the vaginal Gram-positive non-motile rods were known as “Döderlein’s bacilli” for almost a century. Orla-Jensen laid the foundations for a classification based on four genera of lactic acid bacteria: *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus*(69). In 1928 Thomas first described Döderlein’s bacilli as *Lactobacillus acidophilus*(88).

The traditional phenotypic methods that were available, and which are still very important in current classifications, are: morphology, mode of glucose fermentation, growth at certain “cardinal” temperatures (e.g. 10°C and 45°C), and range of sugar utilisation(4). These and other characteristics have not been useful for discriminating the closely related bacteria in the ecological niche of the normal human vagina, which mainly belong to the *L. acidophilus* group(9).

Other earlier studies using the classic phenotypic identification methods demonstrated heterogeneity of the flora, for reviews see Redondo-Lopez and Zhong(76, 96). The most frequently occurring species were *L. acidophilus, L. brevis, L. casei, L. catenaforme L. fermentum, L. jensenii, L. plantarum, L. rhamnosus* and *L. salivarius*. This reflects the unreliability of the phenotypic methods, especially within the genetically close *L. acidophilus* group. One taxonomy of the *L. acidophilus* group was based on DNA homology studies(31, 47).
Modern phylogeny of lactobacilli presents six or seven different groups based on 16S rDNA sequences: *L. buchneri* group, *L. casei* and *L. sakei* group, *L. delbrueckii or acidophilus* group, *L. plantarum* group, *L. reuteri* group and finally *L. salivarius* group(41). DNA-DNA hybridisation as well as phenotypic characters was used by Giorgio (36) for the study of vaginal lactobacilli isolated from asymptomatic women, and these were identified as *L. gasseri*, *L. jensenii* and *L. crispatus*. One not identified group of heterofermentative lactobacilli was found, as well as one isolate of *L. fermentum*.

Antonio et al, using whole-chromosomal probes in a material of 215 American women, found *L. crispatus*, *L. jensenii*, a previously not described species, and *L gasseri* as the dominating species(2). Song found mainly *L. crispatus* and *L. gasseri* in 49 Japanese women using DNA-DNA hybridisation(82). Kilic et al, in a material of 209 women from the US and Turkey, identified most lactobacilli as *L crispatus*, *L gasseri* and *L jensenii*(48).

Since 2000, development of new methods for nucleic acid based comparisons has been rapid. Kullen described a DNA-sequence based comparison of the first third of the 16S rRNA gene for rapid and valid identification of lactobacilli from various human sources belonging to the *L. acidophilus* group(50). In addition, Vasquez et al described TTGE as a useful method for handling identification of large amounts of isolates in studies of the taxonomy of lactobacilli(90). The early results on classification of lactobacilli have later been confirmed by the use of new nucleic acid based techniques targeting the 16S rRNA gene. In a worldwide study of 35 strains from 7 countries, most women harboured *L crispatus*, *L jensenii* and *L gasseri*(71).

There are some important issues that have to be addressed concerning the selection of strains in these studies. Several of the studies were performed on laboratory strains or with the origin of the sample not stated, or without indicating the age, health or hormonal status of the host. Most studies do not define the vaginal samples as normal according to Amsel or Nugent, so it
is not known whether the women had a normal vaginal status or not(1, 66). In most of the studies the colonies were selected by morphology, and in none of the studies was there any discussion of randomisation in the selection of the colonies cultured. All of these factors must have influenced the results, but since all of the studies show almost identical results concerning the species, we must conclude in any case that these three species obviously dominate the normal vaginal microflora.

The recent introduction of techniques for studying the bacterial nucleic acids without previous culture has further expanded our knowledge of the vaginal flora. This provides an option for studying the vaginal flora, including many cases of BV, and, in fact, the focus of the studies pertains to the syndrome of BV. Fredricks analysed some of the first studies using cultivation-independent methods to study the vaginal flora(30). In two studies the flora was characterized with Gram-stained smears, and if normal the dominant species were *L. crispatus, L. jensenii* and/or *L. gasseri*(22, 92). In studies with less well characterized flora, *L. iners* was found as well(44, 97), and as the only lactobacilli in patients with BV according to Amsel(28). A number of uncultivable, previously unknown species in the niche were found and are commented on in the following section on BV.

In an African study using Nugent’s scoring of Gram-stained smears from healthy women attending a reproductive healthcare service, 51% were scored intermediate, 35% normal, and 14% BV. Among the patients without BV, sequencing of a part of the 16S rRNA gene revealed that 65% of the patients harboured *L. iners*(3). These results are consistent with a Japanese study where *L. iners* was found in 40% of women with a normal flora, in 48% with an intermediary flora and in 46% of women with BV according to Nugent’s score(86). In a recent American study of women without signs of vaginal infection, 52% of the patients were dominated by *L. iners*(98).
When trying to delineate normal flora vs. abnormal flora, the patophysiology of the normal microflora and its role in safeguarding against other organisms and overt infection must also be considered. Previously, competitive exclusion of pathogens was considered to be a major task for the normal vaginal flora (5). The acidic milieu that is hostile to many pathogens has also been attributed to the lactobacilli (6, 7). Another major issue has so far concerned the question of whether vaginal lactobacilli are hydrogen peroxide producers or not, stemming from the idea that a hydrogen peroxide producing lactobacillus could be the “normal” and thus a protective lactobacillus (19). Bacteriocins synthesized by the ribosomes to inhibit growth of other bacteria are frequent in lactobacilli (59). In the *L. acidophilus* group they have so far been found only in *L. acidophilus*, which is not frequently found in the normal vaginal flora (70).

Recent papers have introduced new hypotheses on proinflammatory changes in BV (94, 95):

1. The innate immunity seems to be most important. Studies of the normal flora in other niches indicate that TLR of many different types, MBL, and certain heat-shock proteins might be of major importance in normal vaginal flora as well (33). This remains to be studied.

2. The adaptive immune regulation should also be important for protection vaginally. This includes locally produced proinflammatory cytokines, interleukins and other immune modulators (27, 60).

3. The presence of locally produced enzymes like secretory leukocyte protease inhibitor (27), prolidase and sialidase (11).

There is reason to believe that continued studies of host-microorganism interactions will follow these general hypotheses and, in fact, gene polymorphisms in human genes coding for MBL, IL1ra and TLR4 have already been found and the allelic variations are suspected to be of importance for the development of BV (34, 35, 37, 65).
Bacterial Vaginosis

Apart from lactobacilli, the vagina harbours many other bacterial species in varying amounts in conditions that are not considered healthy. In certain conditions the lactobacilli in fertile women are far outnumbered, mainly by anaerobes. The most frequent disturbance is the syndrome of Bacterial Vaginosis (BV)(83). The lactobacilli in BV are overgrown by large amounts of *Gardnerella vaginalis* and anaerobes, mainly *Bacteroides* spp and *Mobiluncus*; for reviews see Hillier and Forsum(25, 43). In the present decade a number of fastidious species have been found in large amounts in the vaginal secretions of women with BV. These include *Atopobium vaginae*(78, 92); *Eggerthella* species, *Leptotrichia* species, *Megasphaera* species and a newly discovered species of the *Clostridiales* order(29, 86, 87, 97).

The prevalence of BV varies widely in different populations. In cervical PAP smear screening programs and in antenatal care units the prevalence is below 10 %(52, 53). In women undergoing termination of pregnancy in Sweden the incidence was found to be 20 %(57), in a recent national survey in the US it was 29%(49) and in rural Uganda over 50%(72).

Just as little is known about how to delineate the healthy vaginal flora from the abnormal vaginal flora, studies have long been hampered by the lack of common understanding of how to recognize and categorize bacterial species that comprise normal and various abnormal vaginal states. This has been troublesome, since the main field of BV research primarily concerns studies comparing randomised controlled treatment of healthy vs. non-healthy groups. When it is impossible to delimit the healthy and non-healthy groups based on proper identification of bacterial types relating to established bacterial species, the conclusions based on the results will be doubtful.

The effects of loss of the normal microflora in BV are associated with several severe reproductive and genitourinary complications in women(55, 67). Preterm delivery is the
leading cause of perinatal mortality and morbidity in the developed world(39, 89). There may be a clinical association between BV and preterm delivery(56, 67, 85), and if treatment of BV could prevent only a few cases of premature births, much pain and high costs could be prevented (VI).

BV has also been associated with an increased risk of postoperative infections after hysterectomy(73) and abortion(54) and may enhance the transmission of HIV(79).

During the last two decades numerous studies have been presented in which different Lactobacillus spp have been introduced into the vagina for the purpose of normalizing the vaginal flora. Maggi used a mixture of *L. brevis*, *L. salivarius* and *L. gasseri*(61). McLean suggested *L. acidophilus*(64) and Ocana *L. crispatus*(68). Falagas presented a summary of randomised clinical trials with *L. acidophilus; L. fermentum and L. rhamnosus* administered orally and *L. gasseri* administered vaginally(20). In a recent study from Norway, patients with BV who had received vaginal treatment with clindamycin thereafter were given supplementary vaginal treatment with *L. gasseri* and *L. fermentum* for three cycles of 10 days each. The time to relapse was marginally prolonged(58).

By and large, no effects or only marginal effects of lactobacillus instillation are documented in these studies and no study has so far been published where the treatment strains have been proven to adhere to and colonize the vagina.

**Diagnosis of Bacterial Vaginosis**

The diagnostic tools for BV are also a matter of controversy. Following the initial discovery of the mixed bacterial overgrowth of mainly anaerobes(32), different scoring systems have been developed. Since BV is not a simple infection caused by a single agent, the aim of using a cultural technique for diagnosis of BV is not achievable in clinical practice due to the
required workload and costs. The strong association between the many newly discovered uncultivable species and BV further illustrates this. The development of PCR probes for detection of the microbes present in the vaginal fluid has made it possible to construct DNA libraries of the microbiology with several microbes that were previously undetected. The lack of knowledge of the microbiology of BV resulted in development of several scoring systems during the final decades of the 20th century. In the early 1980s the composite criteria of Amsel were established as the gold standard for diagnosis of BV. The disadvantages are several. Firstly, practically all of the tests are subjective, i.e. they can differ from time to time even with the same investigator. The pH is not always unequivocal and can be falsely elevated by mixture of the vaginal and cervical secretions. Likewise, the sensitivity for the amines differs depending on whether or not the investigator ate garlic for lunch or, even worse, whether he or she smokes. Some individuals cannot sense the unpleasant odour of trimethylamine due to a genetic aberration (Forsum, U. personal comm.). The microscopic analysis of clue cells is sometimes difficult, and the discharge of a woman with BV can vary considerably. In the US, the clue cell criterion is applied differently, from mere existence to occurrence on 20% of the epithelial cells. For a review see Forsum et al(26). Amsel’s criteria are, however, the gold standard that research in BV has had to put up with for almost 20 years.

In the light of these difficulties, there has been a search for different and hopefully more robust scoring systems. Spiegel et al.(84) defined a scoring system for bacterial morphotypes, which can be seen in Gram-stained smears made from vaginal secretion of women undergoing examination for BV. Nugent et al. (66) later refined the system, and the revised system has gained wide acceptance as the scoring system of choice for research, but not in routine laboratories as it is time-consuming. Other scoring systems, based on similar principles(42, 93) or using wet mounts of vaginal secretion observed in phase contrast microscopy(80), have
also gained acceptance in some parts of the world. A similar system has been proposed for use with PAP-stained vaginal (but not cervical) smears(74). As mentioned above, a key issue is definition of the normal vaginal status. In my studies I have chosen to define the vaginal flora of the patients according to the composite criteria of Amsel(1) and with a smear Gram-stained and analysed according to Nugent’s criteria(66). Only patients who were normal according to Amsel’s criteria and to Nugent’s criteria, i.e. with a score of 1-3, were included in studies II, III and IV.
MATERIAL AND METHODS

Microbiology in vaginal secretions as observed in the microscope
(paper I)

This paper was organized as a workshop for collaborative validation of robust concepts and their dimensions expressed as vaginal smear scores. The workshop was undertaken on the assumption that the concepts (i.e. morphotypes of bacteria, cells or other concepts) that form the best basis of a scoring system are not known with certainty. Specific purposes of the workshop were to evaluate the deviance between participants’ interpretations of each of some selected criteria of BV and to evaluate the agreement between the different criteria assessed as the mean of the different interpretations.

Collection of samples

An invitation to take part in the workshop was sent by the organizers to known researchers and practitioners in the field. Participants collected a total of at least 20 slides of vaginal smears including smears from at least seven patients with BV. The samples were collected from patients seeking care for lower genital tract complaints using the local standard method. A total of 258 slides thus collected were circulated among participants from the US, Europe and Australia.

Amsel’s wet smear composite clinical criteria

The diagnosis is carried out in any clinical setting with access to a microscope and is based on the following four criteria:

1. Typical, thin, milky, homogenous fluor.
2. pH > 4.5
3. Clue cells seen under the microscope in a wet smear
4. Positive sniff test, i.e. an odour like rotten fish immediately after adding a drop of 10% potassium hydroxide to the fluor.

Amsel’s criteria are positive for BV when three of the four criteria are fulfilled(1).

**Nugent’s scoring system for Gram-stained smears**

<table>
<thead>
<tr>
<th>Score</th>
<th>Lactobacillus morphotype /vision field(x1000)</th>
<th>Gardnerella morphotype /vision field(x1000)</th>
<th>Curved bacteria morphotype /vision field(x1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&gt;30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>5-30</td>
<td>&lt;1</td>
<td>1-5</td>
</tr>
<tr>
<td>2</td>
<td>1-4</td>
<td>1-4</td>
<td>&gt;5</td>
</tr>
<tr>
<td>3</td>
<td>&lt;1</td>
<td>5-30</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>&gt;30</td>
<td></td>
</tr>
</tbody>
</table>

In the Nugent scoring system scores of 0-3 = normal flora, scores of 4-6 are intermediary scores, and scores of 7-10 are BV(66).

**Statistics**

The results of different participants were compared using the kappa coefficient. Intra-observer reliability of quantitative ratings was compared to agreement beyond chance. A value above 0.75 represents excellent agreement beyond chance(24).
Characterization of the dominating flora in healthy women with normal vaginal flora (papers II and III)

Study population
In paper II, 23 healthy women scheduled for their regular cervical PAP smear at the antenatal care unit at the University Hospital in Linköping, Sweden, were examined concerning their vaginal microbiological status. In paper III, the dominating vaginal lactobacilli of 23 healthy women were studied. Of the women in the first study, 12 returned three years later for their scheduled PAP-smear check-up and thus took part in both studies.

Collection of samples
One sample of vaginal fluid was collected for culture, one for wet smear and composite criteria of Amsel, and one was air dried for later Gram staining. A sterile cotton swab was rolled over the upper third of the vaginal wall and placed in Copan transport medium. After dilution the specimens were cultured for 42-72 h on horse blood agar and Rogosa agar in 10% CO₂ and 5% O₂ at 37°C. Colonies from each kind of agar were randomly selected by marking colony positions in a random fashion with a fine-point felt-tip pen on the bottom of each plate. After the plate was inverted, the colony closest to the felt-tip pen mark, regardless of colour or size, was picked for propagation and Gram staining. Colonies with Gram-positive bacteria with a Lactobacillus like morphology were chosen for further investigation.
In paper II, the samples were spread with a rubber policeman on three different agar plates, and three colonies were randomly selected from each plate for reculture. The first five samples were cultivated in duplex. For paper III, 10 colonies from Rogosa agar and 10 colonies from blood agar were randomly selected for repropagation.
Secondary Structure: small subunit ribosomal RNA

Lactobacillus acidophilus
(M58802)
1. cellular organisms 2. Bacteria 3. Firmicutes
4. Bacillus/Clostridium group 5. Bacillus/Lactobacillus/Streptococcus group
6. Lactobacillaceae 7. Lactobacillus
July 2001

Citation and related information available at http://www.rna.ccb.b.c.uchas.edu/
Nucleic acid based techniques

Two different types of molecular biology methods were used to group and identify the strains to the species level.

RAPD-PCR is a fingerprinting method targeting the whole bacterial genome.

The other methods all target the 16S rRNA gene, constituting around 1500 nucleotides. The gene has extremely conserved regions, which are suitable targets for primers, mixed with highly variable regions. Most endogenous bacteria as well as most pathogens are possible to characterize with different polymerase chain reaction (PCR) methods. The gene from Lactobacillus acidophilus is illustrated in figure 1(8).

Randomly Amplified Polymorphic DNA-PCR

Randomly amplified polymorphic DNA (RAPD) -PCR analysis was used to group the isolates in the first study. An arbitrary primer of 9 nucleotides was used to amplify the DNA at a low stringency annealing temperature. The number and the location of the random annealing sites vary for different strains of a bacterial species. After separation of the amplified products with agarose gel electrophoresis a pattern of bands characteristic of the particular bacterial strain is produced.. Photographic negatives of the gels were scanned, analysed and grouped by GelCompar 4.2 software.(46, 75, 91)

Temporal Temperature Gradient gel Electrophoresis

A PCR-product from the first 350 nucleotides of the 16S rDNA is analysed by TTGE on a polyacrylamide gel plate with the temperature gradually rising from 61 to 71°C, expanding the separation range and thus increasing the sensitivity.(90)
Multiplex PCR

Multiplex PCR targeting the intergenic spacer region between the 16S and the 23S rDNAs and the 23S rRNA gene is used in order first to group the strains to one of four groups. The strains are then characterized with a multiplex-PCR II with species-specific primers for L. acidophilus and L. jensenii and for L. crispatus and L. gasseri(81). The amplicons are identified with agarose gel electrophoresis.

16S rDNA sequencing

The target for identification was the first 900 nucleotides from the 5’ end. After purification the PCR products are used in sequence reactions with the Thermo Sequenas Cy5 Die Terminator Kit with biotinylated primer. After purification, sequences are determined in an ALFExpressII instrument.

Pyrosequencing

Pyrosequencing is a real time sequence analysis technique. Pyrophosphate is detected upon nucleotide incorporation. Broad-range PCR amplification of 16S rDNA variable regions V1 and V3 is performed with one of the primers in each pair being biotinylated (Figure 1). The biotinylated PCR products are prepared with streptavidin coated Dynabeads followed by denaturation with sodium hydroxide.

1. The sequencing primer is hybridized to the single stranded DNA template and incubated with DNA polymerase, ATP sulphurylase, Luciferase, Apyrase, Adenosine 5’ phosphosulphate (APS) and Luciferin.

2. The first deoxynucleotide is added, incorporated by DNA polymerase. Pyrophosphate (PPI) is released in equimolar quantity to the incorporated nucleotides.
3. \[ \text{PPi} + \text{APS} \xrightarrow{\text{ATP sulphurylase}} \text{ATP} \]

4. \[ \text{Luciferin} + \text{ATP} + \text{O}_2 \xrightarrow{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + \text{CO}_2 + \text{light} \]

5. A CCD-camera detects light which generates a peak on a printer in proportion to the number of incorporated nucleotides. Unincorporated nucleotides and ATP are degraded by apyrase.

6. Back to step 2, with addition of the next nucleotide, and the process starts over again.

The signatures are then classified by alignment with NCBI catalogued sequences.

Characterization of the dominating flora in women during IVF treatment (papers IV and V)

Study population
The study population started with 34 women following the protocol for IVF- treatment with FSH-stimulation and ovum pickup. Five patients who did not show normal vaginal status according to Amsel or Nugent were excluded. Twelve of the patients returned only once and were likewise excluded from the study. Four of these patients with lactobacilli growing only on blood agar are presented separately in paper V. Study IV comprises the vaginal secretions of the remaining 17 women cultured at 62 occasions, three to five times per patient.

Hormonal assessment
Patients were treated according to the national guidelines for hyper ovulation in IVF treatment. After downregulation with a GnRH- analogue, buserelin 0.15 mgx4 nasal inhalation, FSH-stimulation was added (follitropin alfa or beta) with a typical subcutaneous
start dose of 150 IE daily.

The level of oestradiol in plasma was measured two weeks after start of the GnRH-analogue, within one week from start of FSH-stimulation, and at each following visit with an ultrasound check of follicular growth, and finally at ovum pick up. The patients who established a pregnancy returned for an ultrasound check six weeks later and plasma was then also obtained for determination of oestradiol.

**Collection of samples of vaginal secretion**

Samples were collected and cultured in the same manner as described above for studies II and III. The selection of strains was done as described earlier. When growth on Rogosa agar was successful, no colonies were collected from the horse blood agar plates. If colonies grew only on horse blood agar, they were collected.

**Bioinformatics**

PCR and pyrosequencing was performed on the V1 and V3 regions of the 16S rRNA gene in the same way as described above.

Reference 16S rRNA gene sequences of vaginal origin were aligned and arranged in BioEdit(40) with Clustal W and with the Mega3(51) software. The signatures of the V1 and V3 regions were checked for their ability to discriminate to the species level.
RESULTS AND DISCUSSION

Microbiology in vaginal secretions as observed in the microscope

Although Amsel's criteria are the accepted "gold standard" for the clinical diagnosis of BV, the numerical score devised by Nugent et al. has gained wide acceptance as the scoring system of choice. The Nugent scoring system is based on Spiegel's bacterial morphotypes in Gram-stained smears. The use of Gram-stained vaginal smears has also been validated against Amsel's criteria in several different contexts, i.e. studies in the areas of obstetrics and gynaecology. Agreement between the Amsel and Nugent criteria can vary, but by and large the Gram stain appears to be the more accurate method(45). In treatment studies the more reproducible Gram stain scoring methods are thus favoured. It is, however, important to ensure that scoring systems meet the quality assessment requirements for procedures used in daily diagnostic work. From this perspective, the robustness of scoring procedures must be assessed when they are performed in different settings and populations around the world and by different observers. The scoring systems have been criticized for having variables that are not independent of each other and for including variables with low sensitivity and specificity. Furthermore, the Nugent scoring system was originally set up to be used for BV diagnosis in pregnancy.

In the international workshop on BV scoring (I) that is the starting point for this thesis, the results were generally encouraging in that good concordance was observed among most observers when valuated against each other with kappa statistics. However, many disagreements arose as to the relation of scoring systems to diagnostic concepts not related to BV (e.g. altered vaginal flora and intermediate flora), indicating a great need for further
studies on how to standardize and validate the scoring systems used. Specific technical and interpretive items that were pinpointed by the workshop results include:

1. In general, there were major discrepancies when the lactobacilli were few in number. This is of importance since the score intervals are narrow.

2. Disagreement on how to delineate between Gram-positive rods and small type bacteria (i.e., *Gardnerella* and *Bacteroides* morphotypes).

3a. Further difficulties included preparation of specimens. Collecting techniques and tools varied: speculums, wooden tools for PAP smears, cotton swabs and pH testers - resulting in different thicknesses of the slides.

3b. Staining procedures varied - decolourization is the critical step.

4. Variations in vision fields among microscopes. The variation is estimated at a factor of two or three in some microscopes (IX).

In addition, a recent publication pointed out the presence of sub-categories of slides from BV-free women that pinpoint, in particular, a distinct morphotype related to *L. crispatus* and a subcategory possibly related to *Bifidobacterium* spp (98). There is also a lingering feeling among researchers and practitioners using various scoring systems in microscopic slides for the diagnosis of BV that the scores obtained do not always correspond to the perceived condition of the women who are examined, and that this might be due to a variety of interpretational problems.

To the best of our knowledge, no work has previously been published, apart from an initial report by Dunkelberg, in which parameters pertaining to the scoring situation itself, and validation of the scoring of the actual object seen, have been discussed in detail (17).

There has been disagreement on which morphotypes should be considered gram-positive rods and thus scored as the lactobacillus morphotype (XII). A frequently occurring staining phenomenon is the tendency of old lactobacilli to lose their gram-positivity. The staining
procedures vary; small bacteria morphotypes (*Gardnerella* and *Prevotella* morphotypes) may vary in size and exist as round to more elongated forms where there is no defined border to separate them from the lactobacillus morphotypes. No definite criteria exist for distinguishing different *Lactobacillus* morphotypes and for demarcating them from the *Gardnerella* and *Prevotella* morphotypes. Morphotypes suggestive of gram-positive cocci, which are not accounted for in the scoring system, might in some cases be difficult to distinguish from the *Gardnerella* and *Prevotella* morphotypes.

There is hence a need to study the effects of the above stated problems on scoring results. Digital images of samples would offer a possibility to score exactly the same bacterial cells and thus evaluate how various investigators score the different bacterial morphologies and other specimen components. In a new workshop (XII), the participants were presented with digital images at approximately the magnification obtained in a microscope and asked to identify objects.

The workshop organizers identified 22 categories of microbial morphotypes and cellular elements of importance for BV scoring, and selected digital images taken to represent morphotypes and cellular elements that might be identified with low interobserver variation by participants assigning the images to the predetermined classes.

Some morphotypes and cellular elements were deemed simple and others more difficult, depending on the backgrounds of the 11 participants.

The other 10 participants and I categorized objects into the 22 predetermined categories listed below. The results clearly indicated that most participants categorized various *Lactobacillus* morphotypes similarly, but disagreed regarding the kind of *Lactobacillus* it was (wide vs. thin). *Prevotella* and *G. vaginalis* morphotypes were surprisingly often categorized incorrectly. Results at the more finely grained category level were even more discordant.
The conclusion is that the robustness of categorization of the included objects must be considered doubtful, despite the fact that the categorization follows well-established principles and is in common use all over the world. Categories of microbial morphotypes and cellular elements of importance for BV scoring.

<table>
<thead>
<tr>
<th>Lactobacillus</th>
<th>overdecolourized Gram-positive rod</th>
</tr>
</thead>
<tbody>
<tr>
<td>thin Lactobacillus</td>
<td>curved rod-shaped bacterium</td>
</tr>
<tr>
<td>wide Lactobacillus</td>
<td>yeast</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>sperm</td>
</tr>
<tr>
<td>Fusobacterium</td>
<td>leucocyte</td>
</tr>
<tr>
<td>G. vaginalis</td>
<td>cervical epithelial cell</td>
</tr>
<tr>
<td>Mobiluncus</td>
<td>squamous epithelial cell</td>
</tr>
<tr>
<td>Prevotella</td>
<td>unknown object</td>
</tr>
<tr>
<td>coccus morphotypes</td>
<td>artefact</td>
</tr>
<tr>
<td>coccobacillus morphotypes</td>
<td>other object</td>
</tr>
<tr>
<td>Gram-positive rod</td>
<td>stain deposit</td>
</tr>
</tbody>
</table>

In the BV 00 workshop (I), mutual agreement on the three morphotype criteria (Lactobacillus, Gardnerella/Prevotella and Mobiluncus) was lower than agreement on the quantity of any given organism. This finding suggests that there is a smaller factual difference between the criteria. The study also showed excellent interobserver agreements for the weighted kappa statistics for scoring of so-called intermediate flora. Hillier et al. suggested that a higher Nugent score is a sign of aggravation of BV. The data of Verhelst et al. point to the possibility of identifiable subgroups of Lactobacillus morphotypes that correlate to Lactobacillus species present in the slides. However, it has not been adequately determined if the Nugent intermediate score truly represents another clinical group. Further, the relationship to disease
concepts other than BV, such as aerobic vaginitis and altered vaginal flora, has not been
explored in detail. Clearly, more studies are needed to elucidate these relationships. Our
findings indicate that the basic categorization of morphotypes is a key problem that needs
validation if concordance between scoring systems and individuals assigning scores is to be
achieved. This would be facilitated if Gram-stained smears were analysed in detail with
digital images, which is now technically possible with an excellent image quality, and the
respective morphotypes were correlated to individual libraries of DNA sequences of strains in
the vaginal fluid by using the FISH technique(28).

For a mutually agreed upon study of FISH-based DNA probes correlating morphotypes with
dNA sequence differences to be accepted, some basic categorization principles have to be
adhered to: observations in the microscope can be registered using a nominal scale, and
ranked on an ordinal scale(12, 13). The perceived morphotypes can be subjected to
comparative scrutiny, and robustness can be assessed in a comparison of how observations are
made by individuals(14). Definitions of analytical quality specifications are essential.

Dominating normal vaginal flora in healthy Swedish women.

Study II showed that *L. crispatus, L. gasseri, L. iners, and L. jensenii* were the most
frequently occurring species in the healthy vaginas of 23 Swedish women. Twenty of the
women were dominated by one species, and none by more than two.

RAPD analysis directly identified the isolates that could only grow on blood agar as *L. iners*.
*L. iners* is a newly described species(21). The absence of *L. iners* in other studies searching
for lactobacilli might be explained by the use of selective Lactobacillus media such as Rogosa
and MRS agar where these lactobacilli do not grow. The reasons for using the selective media
have traditionally been of a practical nature. Since there are at least 50 different species present in the vaginal fluid, mostly in minute amounts, overgrowth of some of these has made isolation of lactobacilli on routine agars extremely difficult.

Species such as *L. rhamnosus*, *L. fermentum*, *L. plantarum*, and *L. acidophilus* have also frequently been recovered from the vagina (76, 96). The differences in *Lactobacillus* flora between different studies may be attributed to a number of factors. The most likely explanations are variations in the way that samples are taken and treated, the vaginal status, and the fact that identification has previously often been based on phenotypic methods (64, 77).

To summarise, it is necessary to focus on true random sampling of non-selected colonies on non-selective media in healthy vaginal fluid in order to describe the normal flora. An important new finding of this study is the possibility that *L. iners* is one of the normally occurring lactobacilli in the human vagina.

In study III, 17 women were found to be colonized with one species, four by two species and one woman, aged 53 years, by four different species. The species identified were the same as in study II.

Study III has introduced a new, fast, accurate technology that can, at a reasonable cost, provide the tools for a more versatile numerical taxonomy for vaginal lactobacilli. With these new tools the scope of *Lactobacillus* studies can be broadened to include the study of geographical distribution, age, parity, diagnosis, treatment and so on. In such further studies it is also important to define the vaginal status (i.e. normal Nugent and/or Amsel criteria) of the study subjects in order to create a body of knowledge about normal Lactobacilli in fertile women without interference from subjects in the study population with altered microflora such as BV. Further typing of Lactobacillus species is also needed if studies of the epidemiology of the various species in the vagina are to be successful. A possible tool for
identification of subtypes is multilocus sequence typing (MLST)\(^{(15, 62)}\). Such a technique might be necessary in order to trace colonization of administered probiotic strains in the future.

Table I. Study II+ study III

<table>
<thead>
<tr>
<th>Woman</th>
<th>Study II</th>
<th>Species II</th>
<th>Species III</th>
<th>Study III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No of strains</td>
<td>No of strains</td>
<td>No of strains</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>9   G</td>
<td>G</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>27</td>
<td>9   G</td>
<td>G</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>22</td>
<td>18  J</td>
<td>J</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>12</td>
<td>9   J</td>
<td>J10/4G1V1</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>18</td>
<td>8   C</td>
<td>C</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>21</td>
<td>11  C</td>
<td>C12J7</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>23</td>
<td>18  C</td>
<td>C11J2</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>19</td>
<td>3   I</td>
<td>I</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>5   C</td>
<td>G19C1</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>9   G</td>
<td>C</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>15</td>
<td>9   G</td>
<td>J</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>24</td>
<td>6   I</td>
<td>C</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

Table I presents a comparison of the results of studies II and III. Most of the women retained the same dominating species when returning after three years. Four patients changed to one of the other three dominating species. The main results were more or less the same with the two different methods and different numbers of studied strains. The diversity in woman no. 12 might be due to a lower oestrogen level since she was 53 years of age at the time.

**Dominating normal vaginal flora in patients during IVF treatment.**

My thesis work provides more detailed descriptions of the species of lactobacilli that form part of the normal flora in healthy women of childbearing age. The studies also seek to define how the dominant lactobacilli flora changes over time. Women undergoing IVF-treatment can
be used as a suitable model for such studies in that the oestrogen levels of the women are manipulated for the sake of the treatment. Studies on the co-variability of the flora and oestrogen levels are thus possible in these women.

A total of 184 out of 186 lactobacillus isolates were subjected to PCR and pyrosequencing of the 16S rRNA gene regions V1 and V3. Three sequences were obtained for each strain subjected to PCR and pyrosequencing, one for the V1 region and two complementary sequences for the V3 region. All were categorized separately and matched with the reference strain of each species (for *L. jensenii* also two subtypes). Of a possible 552 sequences, 519 were suitable for sequence analysis.

Ten of 17 patients continued to exhibit *L. crispatus*, *L. gasseri* and/or *L. jensenii* with little variation throughout the study period. The flora of three patients was dominated by *L. delbrüeckii*, *L. rhamnosus* or *L. vaginalis*. One patient had a dominance of *L. iners*. The remaining three had an initial flora dominated by *L. rhamnosus* or *L. reuteri*. With rising oestrogen levels, the make-up of the flora changed and became dominated by one of the three species of normal vaginal flora.

Paper V briefly describes four of the patients originally meant to be accepted for the paper IV study. One patient presented with abnormal vaginal flora. At check-up eight days later she presented with normal flora and was thus accepted for the paper IV study. This is patient no. 17, who presented with *Lactobacillus iners* throughout the study period. The other four women, for different reasons, only returned once or twice and were therefore excluded from the paper IV study. Since these patients all had lactobacilli growing only on blood agar, we still found it worthwhile to analyse these strains too, in the same way as the strains in paper IV.

One of the patients showed *Lactobacillus iners* and normal flora in both samples that were taken. Two of the patients presented with Bacterial Vaginosis, were treated with
metronidazole 2g, and returned one week later with normal vaginal flora. The fifth patient presented with abnormal flora and presented with normal vaginal flora at check-up thirteen days later.

When these patients had normal vaginal flora according to Amsel and Nugent, the strains were recultured and three colonies were randomly selected in the same way as described earlier. One of these strains from each sample occasion was analysed with PCR and pyrosequencing as in papers III and IV and was found to be *Lactobacillus iners* in all cases. These findings are in concordance with the results of Ferris et al. (23).

The results from studies IV and V clearly indicate that by using molecular biology tools we are now on the brink of finding ways of mapping the important changes in the vaginal flora that could be the basis for further studies.
General summary

The diagnostic tools available to delineate healthy vaginal flora from non-healthy vaginal flora need to be further developed. Poorly defined vaginal status is at least in part responsible for the discordance of results in studies on the vaginal flora and is furthermore a key issue in treatment studies of Bacterial Vaginosis.

Molecular biology techniques, especially sequencing of variable regions of the well-characterized 16S rRNA gene, are accurate in identifying the previously inseparable species of the genus *Lactobacillus*. Pyrosequencing is suitable and accurate for high throughput identification of vaginal lactobacilli.

The dominant species in healthy Swedish women and in Swedish women during IVF treatment are *L. crispatus*, *L. gasseri* and *L. jensenii*. This is in concordance with studies in very different settings around the world.

*L. iners* can be the dominating species in a healthy flora during the different phases of IVF treatment, and initially after spontaneously resolved or medically treated Bacterial Vaginosis.

*L. iners* does not grow on media selective for Lactobacilli and thus it has not been identified in many studies. To the best of my knowledge it has not previously been reported as dominating the flora of women with normal vaginal flora.

During IVF-treatment at low oestrogen levels, other species of Lactobacilli, normally found in the gut, can be dominant in the vaginal flora. At rising oestrogen levels the flora progressively changes and becomes dominated by one of the three species found in normal vaginal flora.
Acknowledgements

I would like to express my thanks to:

All the women who contributed with small drops of vaginal fluid and without whom this work would not have been possible.

Professor Urban Forsum, my main supervisor, for bringing me back to focus over and over again, for introducing me into the fascinating world of the microbes, and for supporting me in continuing this work and completing my thesis.

Assistant professors Lars Fåhraeus and Per-Göran Larsson, my co-supervisors, for pleasure, friendship, and refreshing, wild, and crazy scientific ideas that finally produced quite a few articles.

My co-authors in Lund and Linköping for excellent guidance, co-operation and skilful production of manuscripts.

My colleagues and all the staff both at the Division of Obstetrics and Gynaecology and the Department of Obstetrics and Gynaecology especially the Antenatal Care Unit, Outpatients Clinic and Reproductive Medicine Centre as well as at the Division of Clinical Microbiology and the Department of Clinical Microbiology in Linköping.

Special thanks to laboratory engineer Maud Nilsson and laboratory technicians Bodil Carlsson and Anita Johansson for support and education in laboratory practice and for answering my never-ending microbiological questions.
My relatives and friends for being there when I needed you.

My beloved wife Evy and our children Frida, Gustav and Arvid for giving me the fighting spirit and for your eternal love.

The study was supported by grants from the Medical Research Council of Southeast Sweden and by the ALF project at the University Hospital Linköping, Sweden.
References


