Characterization of the vaginal microflora in health and disease

Raluca Datcu

Correspondence: Raluca Datcu, Frøhaven 18, 2630 Taastrup, Denmark

E-mail: ralucadudau@yahoo.com

THE 3 ORIGINAL PAPERS ARE

1. Datcu R, Gesink D, Mulvad G, Montgomery-Andersen R, Rink E, Koch A, Ahrens P, Jensen JS. Vaginal microbiome in women from Greenland assessed by microscopy and quantitative PCR. BMC Infect Dis 2013 Oct 16; 13 (1):480.

2. Shipitsyna E, Roos A, Datcu R, Hallen A, Fredlund H, Jensen JS, Engstrand L, Unemo M. Composition of the vaginal microbiota in women of reproductive age – sensitive and specific molecular diagnosis of bacterial vaginosis is possible? PLoS One 2013 April 9;8(4):e60670.

3. Datcu R, Gesink D, Mulvad G, Montgomery-Andersen R, Rink E, Koch A, Ahrens P, Jensen JS. Bacterial vaginosis diagnosed from first void urine specimens. J Clin Microbiol 2014 Jan;52(1):218-25.

BACKGROUND

Historical overview

In 1894 Döderlein described that the typical Döderlein's rods were missing in women who complained of a thin, greyish-white discharge and these rods were replaced by bacteria of different morphologies (31). The condition was later called "nonspecific vaginitis" and the aetiology was unknown when sexually transmitted infections (STIs) were excluded. Döderlein's bacilli were identified later as lactobacilli, and they produce lactic acid by degradation of glucose released by the vaginal epithelial cells and are responsible for the low pH of the vaginal fluid of reproductive-age women and for maintaining a healthy vaginal environment (Figure 1).

Figure 1:
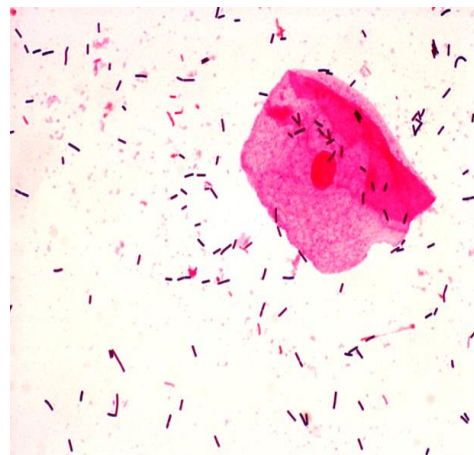
Gram stained vaginal smear with Lactobacillus spp.

G. vaginalis was originally discovered by Leopold in 1953 who described it as a novel *Haemophilus*-like species associated with prostatitis and cervicitis (96). In 1954 and 1955, Gardner and Dukes hypothesized that nonspecific vaginitis was caused by an STI with a Gram-labile rod, first named *Haemophilus vaginalis* (52,53), as it was for the first time linked with nonspecific vaginitis, then *Corynebacterium vaginale* (178). This bacterium was later shown to represent a new genus, *Gardnerella*, and was named *G. vaginalis* (127). The role of *G. vaginalis* in the aetiology of BV could not be proven, but the key feature of the diagnosis was the presence of vaginal epithelial cells heavily covered with bacteria in wet-mount microscopy, "characteristic granular appearance" – the so-called "clue-cell" with "indefinite outlines" (53). The vaginal cells maintain this appearance on Gram-stained vaginal smears (Figure 2).

Figure 2:
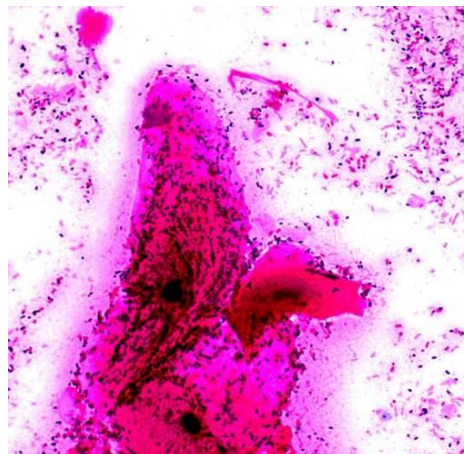
Gram stained vaginal smear with BV and clue-cell.

In 1983-1984, nonspecific vaginitis was termed bacterial vaginosis (BV), characterized by absence of specific STIs, overgrowth of Gram-negative anaerobes of various morphologies and the hypothesis that certain anaerobes and/or *G. vaginalis* may cause BV was launched. Gas-Liquid Chromatography showed that in normal vaginal fluid, lactate is the predominant acid and the predominant organisms were *Lactobacillus* and *Streptococcus* spp., while in nonspecific vaginitis lactate was decreased and succinate (produced by anaerobes), acetate (produced by *G. vaginalis*) and butyrate (produced by *Peptococcus* species) were increased; a high ratio of succinate to lactate proved to be a useful indicator in the diagnosis of BV (148,150).

In 1983, the diagnosis was standardized, based on clinical criteria introduced by Amsel (3) and on Gram stained smears developed by Spiegel (149).

Nugent further standardized the BV diagnosis as a scoring system from 1-10, where 0-3 represented normal flora, 4-6 intermediate and 7-10 bacterial vaginosis flora (120). Nugent's scoring system was based on counting three morphotypes: *Lactobacillus* spp., *G. vaginalis* or bacteroides (small gram-variable rods or gram-negative rods) and curved Gram-variable rods. In order to study the association between late miscarriage and preterm delivery and BV, Hay used the criteria for diagnosing BV on Gram-stained smear introduced by Spiegel (149) with the addition of an intermediate category (62). These criteria were based on relative quantities and defined as: grade I, normal (predominantly *Lactobacillus* morphotypes), grade II, intermediate (reduced *Lactobacillus* mixed with other morphotypes) and grade III, abnormal (few or no *Lactobacillus* morphotypes with greatly increased numbers of *G. vaginalis* or other morphotypes, or both). Preterm deliveries and late miscarriages occurred more often in women diagnosed with BV in early pregnancy (62). Later, in 2002, Ison and Hay developed and validated a quick Gram-stained smear diagnosis for BV for STI clinics, by using the three grades previously mentioned and by introducing two additional grades: grade 0, epithelial cells with no bacteria, and grade IV, epithelial cells covered with Gram positive cocci only (77). Based on Ison-Hay's criteria, a more detailed scoring system was suggested, the so-called Claeys' criteria, which divided Ison-Hay's grade I into 1a (short and thick *Lactobacillus* spp.), 1b (long and thin *Lactobacillus* spp.) and 1ab (mixed *Lactobacillus* spp.) (166). Besides, grade 1-like was defined as consisting of Gram-positive rods, small, short or irregularly shaped (with clubbing and curved edges), often arranged like Chinese letters ("diphtheroid cell types"). Claeys' scoring system indicated in 2007 the association of BV-like flora (grade 2 and 3) or 1-like or 1-purulent with preterm birth, where grade 1-purulent described "idiopathic purulent grade I" smears, i.e. smears showing heavy leucorrhoea with more than 50 PMN per high power field in the presence of normal numbers of *Lactobacillus* spp., but in the absence of pathogens such as Candida spp. (171).

Phase-contrast microscopy of wet mounts for BV diagnosis started to be used initially as part of the clinical criteria (composite criteria) for diagnosing BV (3) and subsequently as the main method (94). Thus, in 1999, Donders classified the vaginal flora on fresh smears based on the relative quantities of lactobacillary morphotypes in relation to other morphotypes (34). In 2000, Schmidt and Hansen introduced a scoring system for wet-mount microscopy for diagnosing BV, taking into consideration lactobacillary morphotypes and small bacterial morphotypes in a quantitative manner (134).

There are studies with controversial results regarding the correlation between the three diagnostic methods of BV: Amsel's clinical criteria, wet mount microscopy and Gram stained smear. Larsson *et al.* demonstrated that clue-cells in rehydrated, air-dried, stored smears examined one month later correlate with the composite clinical diagnosis of BV with a sensitivity of 96 % and a specificity of 98 % (94). A low correlation, however, has been found between wet-smear clue-cells and Gram-stained clue-cells, as 19% of those with Gram stain criteria for BV lacked clue cells (43) and it was speculated that Gram-staining destroys or washes away the bacteria from the clue-cell and that the correct diagnosis of BV should be based only on bacterial morphotypes (163). The BV prevalence determined by Schmidt' and Hansen's wet mount microscopy method agreed more closely with that determined by using Amsel's criteria than with the evaluation by Nugent's criteria, suggesting that wet mount method may be a more suitable alternative to the composite clinical diagnosis than the Gram stain (134). A possible explanation of this agreement is that Amsel criteria consist of a set of four characteristics, among which three are clinical (homogeneous, white vaginal discharge, fishy odour of vaginal discharge before or after addition of 10% potassium hydroxide and pH of the vaginal fluid elevated to over 4.5) but one is based on the presence of clue-cells as detected by wet mount microscopy. On the other hand, an international workshop on vaginal smear-based diagnosis of BV showed high interobserver reproducibility of interpretations between Nugent's, Ison-Hay's and wet smear scores for the diagnosis of BV (47).

Two other pathologic but less known vaginal flora changes have been described and they were related to specific changes of the *Lactobacillus* flora. Lactobacillosis (Figure 3) is a condition characterised by the presence of long lactobacilli and clinically by discharge and discomfort, occurring 7-10 days before menses (75). The cause of this morphologic transformation is unknown and the condition can be effectively treated with antibiotics (75).

Figure 3:
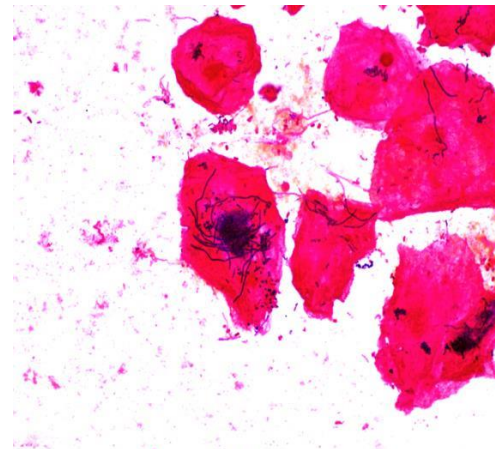
Gram stained vaginal smear showing lactobacillosis.

Cytolytic vaginosis (Figure 4) is a condition present in women who complain of thick or thin white cheesy vaginal discharge with pH = 3.5 - 4.5, pruritus, dyspareunia, and a cyclic increase in symptoms that are more pronounced in the luteal phase (85). Extreme acidity is damaging the epithelial cells (epitheliolysis) and due to the large amount of lactobacilli it is also called "supernormal flora" (38). Treatment consists of sodium bicarbonate douching with the purpose of increasing the vaginal pH (23,85).

Figure 4:
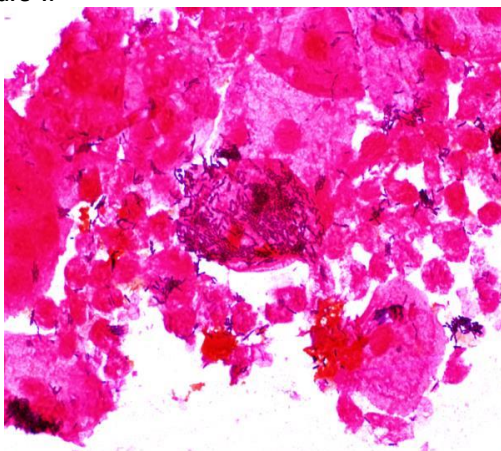
Gram stained vaginal smear with epithelial cell covered with lactobacilli (false clue-cell) and vaginal epithelial cells in fragments (cytolysis).

In contrast to bacterial vaginosis, characterised by the presence of a multitude of anaerobic bacteria, Donders described in 2002 a condition called aerobic vaginitis (AV), in which facultative aerobic bacteria dominate (Figure 4) (35,38). AV on wet mount smear is characterized by a decreased number of lactobacilli, presence of cocci or coarse bacilli, presence of parabasal cells and/or vaginal leucocytes and especially "toxic" leucocytes, which can only be seen on wet mount smear and believed to be packed with lysozyme granules. There are also macroscopic signs of inflammation when examining the vagina of women with AV. Vaginal micro-organisms associated with AV are mainly group B streptococci, *Staphylococcus aureus* and *Escherichia coli*. Desquamative inflammatory vaginitis is equivalent to severe AV described by Donders; it responds to intravaginal treatment with 2% clindamycin (147) and its pathogenesis remains speculative (51,147). Donders further stated that if Gram stained vaginal smears were used, the majority of patients with AV would fall into the group with intermediate flora with Nugent 4-6 (38) based on Hillier's study (69), which described that patients with Nugent 4-6 (intermediate) had increased rates of group B streptococci, *Trichomonas vaginalis* and an abnormal vaginal discharge. Donders also reported that abnormal vaginal flora (disappearance of lactobacilli), AV and BV, present during the first trimester of pregnancy were associated with preterm birth (36,37).

Figure 5:
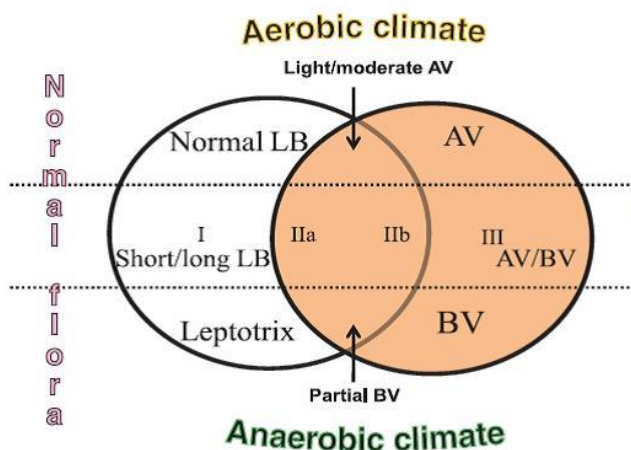
General overview of vaginal bacterial flora types. From left to right the progressive deterioration of the lactobacillary flora is demonstrated. Lactobacillary grades (LBG): I numerous pleomorphic lactobacilli, no other bacteria; IIa mixed flora, but predominantly lactobacilli; IIb mixed flora, but proportion of lactobacilli severely decreased because of an increased number of other bacteria; III lactobacilli severely depressed or absent because of overgrowth of other bacteria. Flora types that thrive in an aerobic climate are presented in the upper layer; flora types that thrive in an anaerobic climate are presented in the lower layer. From: (33).

Starting in 2007, when Fredricks introduced BV diagnosis by species-specific PCR (50), a number of studies have worked with this approach (17,110) and so has the present thesis. A close relation between *A. vaginae* demonstrated by PCR and BV has been found in several studies (156,157,165,170) and sequencing of DNA amplified by 16S broad range PCR from vaginal swabs have confirmed these findings (130,142,151) (Study II). Deep sequencing as well as qPCR with a large panel of species offers the possibility of drawing a detailed, quantitative picture of the vaginal flora, dividing BV communities into clusters and subclusters, dominated by single or grouped BV bacteria. As a new approach, Study III investigated the possibility of diagnosing BV from urine by using qPCR for BV bacteria.

AETIOLOGY, SEXUAL TRANSMISSION & RISK FACTORS
AETIOLOGY
Many researchers prefer not to consider BV as an infection, as inflammation is lacking (Gardner and Dukes described the absence of leucocytes in 1955), but rather as a complex microbial imbalance with indigenous lactobacilli playing a major role (59) and that is the reason why the name of the condition is appointed with the suffix "osis" and not itis".

G. vaginalis
The hypothesis that *G. vaginalis* was the cause of nonspecific vaginitis could not fulfil Koch's postulates, as culture studies showed that *G. vaginalis* could be isolated from women who had no clue-cells on vaginal smear or who were asymptomatic (40,173). Ison found that 80 % of patients with symptoms and

positive microscopy had *G. vaginalis* by culture, but so did 65% of symptomatic patients with negative microscopy (76). Figures as high as 85% for women with Nugent I were found for *G. vaginalis* with the use of sensitive, molecular-based techniques (179) and in the present thesis 97% of women with Nugent I were positive (Study I). It has been discussed whether *G. vaginalis* is the aetiological agent of BV (the mono-microbial hypothesis) or if *G. vaginalis* acts in concert with other bacteria, primarily anaerobes, to produce the disease (the poly-microbial hypothesis) (80). Support for the mono-microbial hypothesis is given by the finding that certain biotypes of *G. vaginalis* positive for lipase activity with oleate as substrate were associated with BV; furthermore, acquisition of BV was associated with acquisition of a new biotype of *G. vaginalis*, and sexual partners harboured the same strains of *G. vaginalis* (8,41,154). Also *G. vaginalis* produces a biofilm found in abundant quantities in BV and this bacterium appears to be an obligatory component of the biofilm in combination with other bacteria (156).

Lactobacillus spp.

H_2O_2 producing vaginal lactobacilli (LB$^+$) are reported to be essential for sustainment of a healthy vaginal microbiota and it is tempting to suggest that BV is caused by the lack of H_2O_2-producing *Lactobacillus* spp. (42,68); however, this cannot necessarily implicate causality, as LB$^+$ were also present in women with BV (42). Some authors have concluded that the production of H_2O_2 by vaginal lactobacilli is protective against BV, as absence of H_2O_2-producing lactobacilli increased the risk of BV acquisition by 2.2 fold (58). Thus, it was speculated that development of effective *Lactobacillus* suppositories with LB$^+$ would maintain or reconstitute a healthy vaginal flora. Hallen and colleagues treated 28 women with BV with lyophilized H_2O_2 producing *Lactobacillus acidophilus*, and compared with 29 women who were given placebo in a double-blind manner (55). They showed an increase in colonization of lactobacilli for 16 women in the treated group in comparison to none in the placebo group; however, most women relapsed into BV after the subsequent menstrual period. The question that arises is that of immune tolerance of exogenous LB$^+$, i.e. if immune tolerance is organism specific, then therapy with exogenous LB$^+$ may not be a successful strategy, triggering an inflammatory response (59). Another approach using acidification and/or hydrogen peroxide treatment to assist lactobacilli during vulnerable circumstances like menstruation or after unprotected sexual intercourse has been suggested (16,176), but this needs further study in randomised controlled studies.

Bacteriophages

Bacteriophages have been proposed as the cause of decrease in vaginal lactobacilli (after studies *in vitro*) and it was shown that prophage carriers (lysogens) were more common among strains derived from BV-affected women (83,122). If bacteriophages indeed play a significant role in BV aetiology, then the possibility of therapeutic use of phage-resistant strains of lactobacilli is worth investigating (7).

Innate immune system components

Vaginal innate immunity in response to microbial perturbation is not fully understood and could be essential for protection from adverse outcomes. Concentrations of IL-8 and IL-1β were highly correlated with counts of neutrophils in vaginal fluid of 60 healthy women and 51 women with BV (both groups non- pregnant), although the IL-8 concentrations and the number of neu-

trophils were not significantly different between healthy controls and women with BV; BV positive women showed a 19-fold increase in the IL-1β concentrations, although most BV positive women did not show any inflammatory sign (20). The cases had abnormal vaginal flora only caused by BV as presence of yeasts and STIs represented exclusion criterion in this study. Cauci and colleagues selected further only women with a high number of neutrophils (≥ 75th percentile, average of 13.9 neutrophils on five fields) and among these women, the percentage of strong IL-1β responders was much higher in the BV group than in the healthy controls. Thus, the measurement of IL-1β concentrations in vaginal fluid may discriminate subgroups of BV positive women with impaired versus activated local immune response (20). Donders showed that there was an increased local production of IL-1, IL-6 and IL-8 associated with AV in pregnancy and that IL-1β is produced much more in women with AV than in those with BV, but the level of IL-6 was even higher, explaining the fact that AV and its triggering of an inflammatory response was associated with an increased risk of preterm delivery, chorioamnionitis and funisitis of the fetus (33). The association between vaginal infection with increased vaginal and amniotic fluid concentrations of IL-6 and IL-8, and chorioamnionitis, preterm prelabour rupture of membranes (PPROM) and preterm birth has been demonstrated previously (72,107,132).

SEXUAL TRANSMISSION

Bacteria such as *Gardnerella*, *Mycoplasma* and *Mobiluncus* could be recovered from urine and urethral scrapings of male partners of women with bacterial vaginosis (53,126), but colonization with BV-associated bacteria was not more frequent in the urethra of sex partners of women with BV than it was in the sex partners of women without BV and the spread of these organisms to the vagina from the rectum seems more likely (74); however, carriage rates of *G. vaginalis* are significantly higher in heterosexual men than among men who have sex with men (MSM) (27). A recent study showed that some BV-associated bacteria (*Atopobium, Megasphaera, Mobiluncus, Prevotella* and *Gemella*) can be detected in coronal sulcus specimens from both sexually experienced and inexperienced men (119).

BV has the same epidemiological features as those of STIs (45) but even though there was evidence that concomitant antibiotic treatment of the woman and her male partner improved the initial cure rates in one study (112), this was not confirmed in many other studies and partner treatment does not decrease BV recurrence rates (24,164,172).

Bump & Buesching and Vaca *et al.* found no significant difference between sexually active and virginal adolescent girls with regard to BV (14,162), while BV was found less commonly in virgins in several other studies (3,46,114) suggesting that an element of sexual transmission is essential in the development of the condition. Use of condoms provides slight protection against acquisition of BV and so does male circumcision (167,172). Prevention against STIs is also worth considering, as it has been shown that BV is associated with *C. trachomatis, N. gonorrhoeae, T. vaginalis* infection and HIV (25,26,117,175). Schwebke and Desmond have shown in a prospective randomized trial that treatment with metronidazole for asymptomatic BV was associated with significantly decreased rates of *C. trachomatis* acquisition, even though the sample size was limited (136); however, causality between BV and acquisition of STIs remains to be established. Study I of the present thesis did not find a significant relationship

between STIs taken separately and BV.

Eren showed that sexual partners harbour the same strains of *G. vaginalis* (41) and women who acquire BV are more likely to have *Gardnerella* strains with different biotypes than women who had a normal vaginal flora at their follow-up visits (8). It is suggested that these special biotypes of *G. vaginalis* are more pathogenic and capable of forming biofilm and these strains are found in women with BV (156).

In conclusion, sexual intercourse plays an important role in BV transmission, BV being "a sexually enhanced disease" rather than a "sexually transmitted disease" (167,172).

RISK FACTORS

BV prevalence varies with age, race or ethnicity, education and socio-economic factors.

Some studies found differences in the prevalence of BV with age, 20% in women 14-19 years old and 30-35% in those aged 30-34 years and older (115); this was not confirmed in Study I of the present thesis.

Race and ethnicity have an impact on BV prevalence. BV is more common among black, non-Hispanic and Mexican-American women than among white, non-Hispanic women (1). African women have a high prevalence of BV (13) with rates comparable with those found in Greenlandic women (116) (Study I). Genetic and geographic factors (as women within the same geographic area generally have same traditions with regard to i.e. sexual and hygienic habits such as douching) may partly explain these differences. It has been shown that Hispanic and black women have higher vaginal pH as compared to Asians and Caucasians and it has been hypothesized that a high Nugent score in asymptomatic women may represent a separate group of normal flora (community group IV), which was overrepresented in Hispanic and black women as compared with Asian (130). Low level of education and low socio-economic status are also associated with BV (1,30). Allsworth & Peipert controlled for sexual history, number of male sexual partners and douching in the past 6 months and stratified education and poverty/income ratio across race or ethnic groups. The study by Desseauve *et al.* included pregnant women and was more prone to confounding, as only age, cigarette smoking, educational level and history of preterm delivery were recorded.

Cigarette smoking increases the risk of BV, and it has been speculated that certain cigarette carcinogens concentrates in cervical mucus and strongly promotes a lytic phase of phage infection killing the protective lactobacilli (7,123).

Vaginal douching confers an increased risk of developing BV (relative risk = 1.21) when confounding was controlled by methods that adjusted for douching behaviour prompted by BV symptoms (12).

Antibiotic treatment for another condition, young age of sexual debut, acquisition of a new sex partner, a recent history of multiple sex-partners and unprotected sex are well-known risk factors for BV (46,172). Surprisingly, however, even though the Greenlandic women taking part in Study I had a high median number of lifetime male sexual partners (12.5), we could not confirm an association between lifetime number of sexual partners and BV in this population.

Women using an IUD have an increased risk for BV, while women using oral contraceptives are more protected against BV, though further studies are needed (15,100). Postmenopausal women in HRT are more protected against abnormal vaginal flora (score 4*, defined as absence of lactobacilli without presence of BV associated bacteria) than postmenopausal women without HRT (19).

BV often relapses around the time of menstruation, probably because of the increase in vaginal pH, due to menstrual blood and hormonal changes (60,61,82).

The rectum has been shown to be a reservoir of BV-associated bacteria and vaginal BVAB may be acquired from extra-vaginal reservoirs and the change in vaginal pH may pave the way for re-colonization from the rectal site (105).

Lesbians (WSW) have a higher prevalence of BV, between 24-51%, and they share the same strains of lactobacilli as sexual behaviours transmit vaginal fluid and also lactobacilli between the women (103). *L. crispatus* was found to be the most commonly shared species, followed by *L. gasseri*; presence of *L. gasseri* was associated with specific sexual practices that could have facilitated its introduction from a rectal reservoir into the vagina (e.g. via digital-vaginal sex) and with a significantly increased risk of BV (104). Vaginal colonization with BVAB 1, 2, 3, *Peptoniphilus lacrimalis* and *Megasphaera* type 2 at diagnosis was an independent risk factor for persistent BV after standard antibiotic therapy, but no specific sexual practices with either male or female partners in the month after treatment predicted either persistent BV or abnormal flora (106).

## CHARACTERISTICS OF BACTERIA FOUND IN THE VAGINAL FLORA

Lactobacillus spp. are Gram-positive rods, but occasionally, some species have aberrant staining properties, particularly when studied from culture-grown organisms. *L. crispatus, L. jensenii, L. gasseri* usually stain as Gram-positive rods from pure culture but may become Gram-variable in staining of culture grown organisms (Figures 6, 7, 8 and 9). *L. iners* usually stain as Gram-negative rods which may be the reason for the late discovery of the importance of this species.
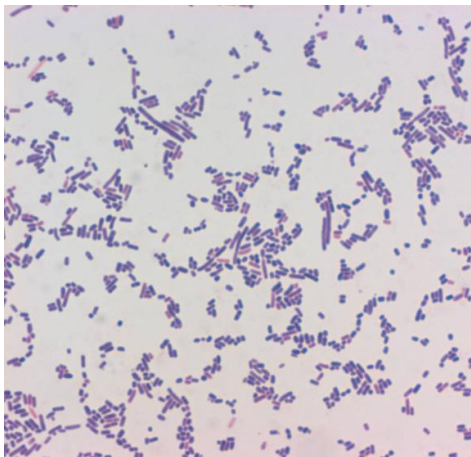
Figure 6:
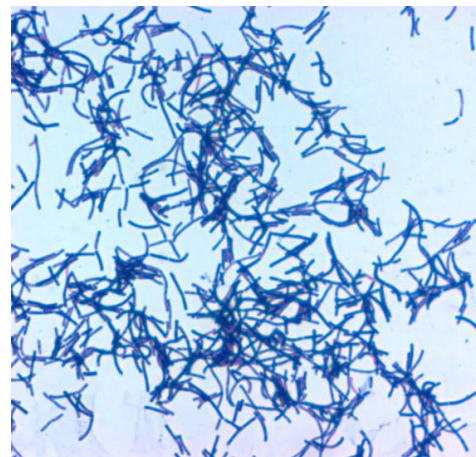
Gram stain culture of L. crispatus – reproduced with permission from Rita Verhelst

Figure 7:
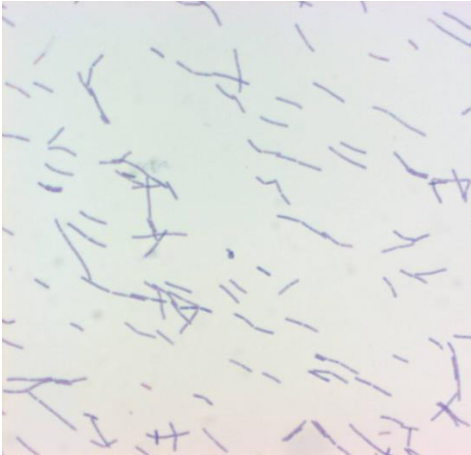
Gram stain culture of L. jensenii – reproduced with permission from Rita Verhelst
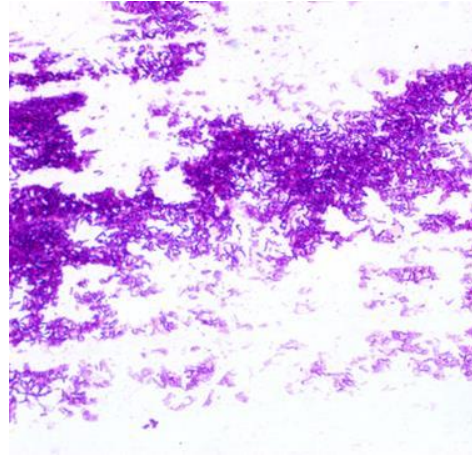
Figure 8:

Gram stain culture of L. gasseri - reproduced with permission from Rita Verhelst
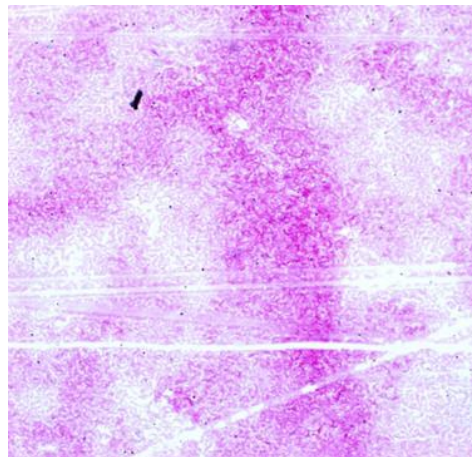
Figure 9:

Gram stain culture L. iners - reproduced with permission from Rita Verhelst

Figure 10:

Gram stain of G. vaginalis in culture.

Figure 11:

Gram stain of Mobiluncus spp.

G. vaginalis has the structure of a Gram-positive bacterium and stains partly or entirely as Gram-positive rods as seen in pure culture (Figure 10) but in vaginal smears it may appear Gram-negative, may remain Gram-variable, but also Gram positive (18). It is fastidious in its nutritional and growth requirements. Susceptibility of *G. vaginalis* to metronidazole is improved if anaerobic incubation is performed instead for aerobic incubation with MIC decreasing 12 times for the former (18); this characteristic may be important for treatment as the vaginal milieu is characterized by a low oxygen tension. *G. vaginalis* is highly susceptible to β-lactam drugs, erythromycin, azithromycin, clindamycin, and streptomycin. MICs for tetracycline are variable from 0.5 μg/ ml or lower to 32 μg/ ml or above, while sulphonamides are ineffective against the bacterium (18).

Mobiluncus spp. are Gram-positive, anaerobic rods, but in pure culture (Figure 11) and in vaginal smears they appear as Gram-variable to Gram-negative curved rods. *Mobiluncus* spp. are susceptible to β-lactam antibiotics (penicillin, ampicillin-sulbactam, cefoxitin, imipenem) and clindamycin and resistant to metronidazole (6).

Different Gram-positive cocci are found in the vagina and they

usually belong to *Peptococcus, Peptostreptococcus, Parvimonas, Veilonella* or *Streptococcus* species (Figure 12).

Figure 12:

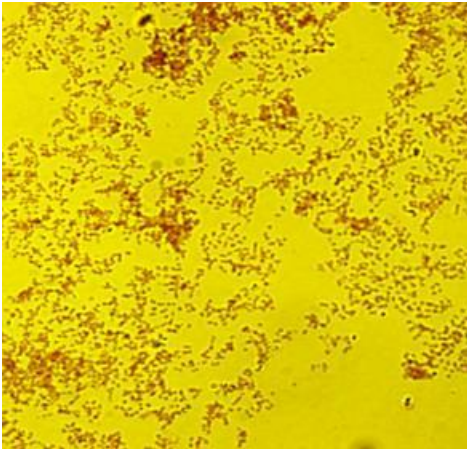*Gram stained vaginal smear with cocci.*

A. vaginae is represented by facultatively anaerobic small Gram-positive elliptical cocci or rod-shaped organisms in smears and in pure culture (Figure 13 and 14, respectively). As it produces lactic acid, it was originally identified as *Lactobacillus* sp. A large proportion of *A. vaginae* strains are metronidazole resistant and clindamycin has higher activity against *A. vaginae* than metronidazole (29,44).

Figure 13:
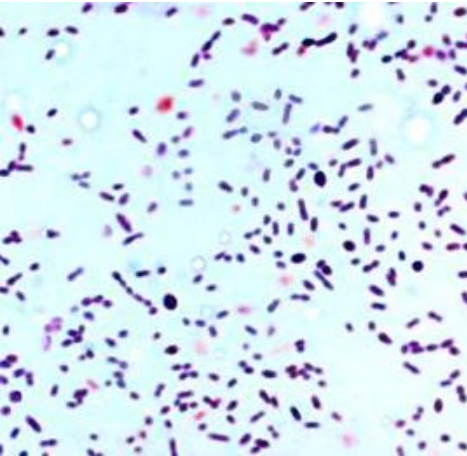
Gram stain of vaginal smear with A. vaginae - reproduced with permission from Rita Verhelst.

Figure 14:

Gram stain of vaginal smear with A. vaginae culture - reproduced with permission from Rita Verhelst.

Prevotella is a genus of Gram negative, pleomorphic, non-motile rods, earlier named *Bacteroides* sp. *Prevotella* is susceptible to metronidazole, and it has been shown that previous therapy with clindamycin increases significantly the resistance to this antibiotic for *P. bivia* and black-pigmented *Prevotella* spp. (*P.intermedia, P. corporis, P. denticola, P. loescheii, P. melaninogenica* and *B. levii*) (5).

Figure 15:
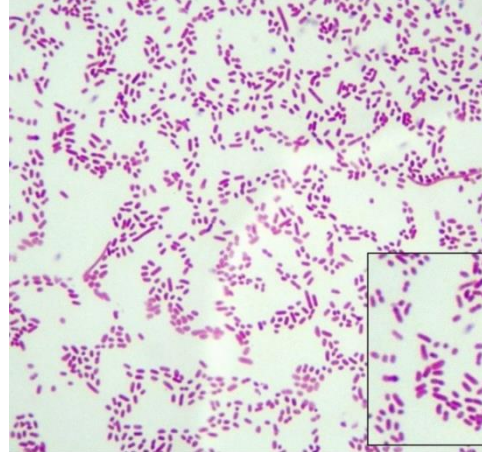
Prevotella sp. (From www.bacteriainphotos.com, M. Balejova)

BVAB 1 and 2 have not been isolated by culture but they appear rod-like in FISH staining with labeled oligonucleotide probes targeting bacterial rRNA (48).

BVAB 3, tentatively named *Mageebacillus indolicus* (Figure 16 and 17) is a slow growing obligate anaerobic, non-motile Gram positive rod with blunt or tapered ends and is asacchrolytic, indole positive, urease negative and is negative for esculin and gelatin hydrolysis and lecithinase and lipase activity. There was no demonstration of spores following heat and ethanol treatments. In smears it most often appears as Gram-variable rods.

Figure 16:
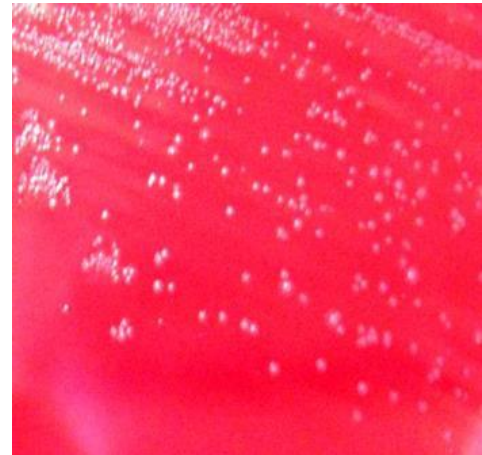
Colony morphology of BVAB3 on Brucella agar supplemented with 5% sheep blood after anaerobic incubation for eight days - the picture is reproduced from poster IDSOG 2008 with permission from Sharon Hillier.

Figure 17:
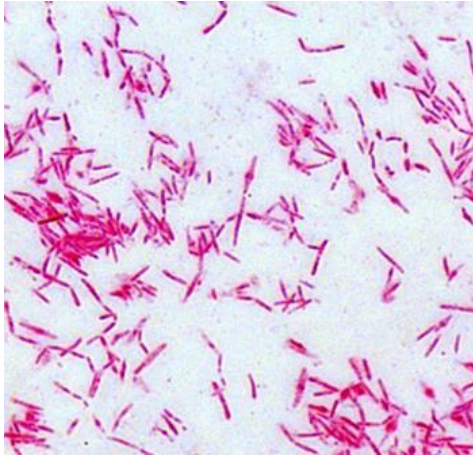
Gram stain morphology of BVAB3 cultivated on Brucella agar - the picture is reproduced from poster IDSOG 2008 with permission from Sharon Hillier.

PATHOGENIC MECHANISMS

There is a normal white blood cell count in the vagina of women with BV microscopically, and when examining the discharge, this is non-purulent (53,73).

BV is characterized by a change in the vaginal flora. A single bacterial species that would cause this shift has not been identified, although *G. vaginalis* is described as having a major role (80). Whether the global overgrowth of anaerobic and facultative anaerobic bacteria in itself is the cause of BV or it is the consequence of a yet unknown event that leads to BV development is unclear. Thus, the aetiology of BV has not been revealed despite considerable research efforts, but a recent trend in the literature is to understand BV as a syndrome with several causes and to classify BV into subgroups with different clusters of bacteria dominating the vaginal flora (130,151) (Study I).

An important pathogenic mechanism could be based on a synergistic relationship between microorganisms associated with BV. For example, when *M. hominis* persists in the vagina after therapy with metronidazole, it is significantly associated with the presence of *Bacteroides* spp., suggesting a possible commensal symbiosis between these two organisms (86). Putrescine produced by some anaerobic BV bacteria stimulates the growth of *M. hominis* and may be one factor leading to the association (146). *G. vaginalis* is often isolated from the female genital tract together with anaerobic organisms; anaerobes produce amines that raise vaginal pH and stimulate growth of *G. vaginalis* (21,148). Commensal symbioses have been described between *Prevotella bivia* and both *G. vaginalis* and *Peptostreptococcus anaerobius* (128,129). A core group of three organisms: anaerobic Gram negative rods (*Bacteroides* sp.), *G. vaginalis* and *M. hominis* has been significantly associated with BV in pregnant women and women who have this group of bacteria are at higher risk for preterm birth and intraamniotic infection (67,87,160).

Another pathogenic path can be explained by control mechanisms of the vaginal microbiome by lactobacilli. *G. vaginalis* is inhibited by lactobacilli and a healthy vaginal environment is maintained by their production of lactic acid leading to a low vaginal pH (139).This production of lactic acid is maintained by oestrogen stimulation leading to an increase of glycogen in vaginal wall. The glycogen is subsequently broken down into glucose, feeding the lactobacilli. Hydrogen peroxide (H_2O_2) mediated antibacterial activity has received significant attention and it has been shown that absence of H_2O_2-producing lactobacilli (LB^+) increases susceptibility to overgrowth of anaerobes and facultative anaerobes (42,58). An inverse relationship between LB^+ and BV has been observed *in vitro* (84,118); however, given the low oxygen tension or even anaerobic conditions in the vaginal environment, it has been questioned whether hydrogen peroxide can be produced under natural conditions (64).

The production of inhibitory substances by vaginal microorganisms, i.e. antibiosis (antagonism) primarily between lactobacilli and other vaginal bacteria may also be a control mechanism for the growth of vaginal populations (102,118,145). Bacteriocins produced by some *Lactobacillus* strains have been found to play a role in inhibition of growth of *G. vaginalis*. Aroutcheva found that 80% of 22 tested *Lactobacillus* strains produced a bacteriocin that inhibited the growth of *G. vaginalis* in vitro (4). Simoes showed that the growth of 78% of 36 clinical isolates of *G. vaginalis* were inhibited by a bacteriocin-producing *L. acidophilus* strain and characterized these *G. vaginalis* isolates as bacteriocin-susceptible (144). Kalyoussef recently found that *Lactobacillus* proteins distinct from bacteriocins may contribute to the bactericidal activity against E. coli. These proteins were identified mostly as surface proteins of *L. crispatus* further confirming the role of this species in maintaining a healthy vaginal microbiome (81). Also, human antimicrobial peptides may play a role as it has been shown that in pregnant women, BV is associated with lower vaginal concentrations of beta defensin 3, but not beta defensin 2 or alpha defensins 1-3 (113).

Biofilm formation may be an important step both in the pathogenesis but also in the persistence of BV. *G. vaginalis* is presumably the first species to adhere to the vaginal epithelium and then provides the scaffolding to which other species adhere (121,168). Not all strains of *G. vaginalis* form biofilm and adherence requires contact with epithelial cells, suggesting that commensal *G. vaginalis* isolates have impaired adherence (57). *Gardnerella* has been found in cohesive and dispersed forms (154) and the biofilm (cohesive form) can be found in urine sediments of both women and men (155) and cohesive *G. vaginalis* strains covering epithelial cells in cryopreserved semen samples have been demonstrated. Thus, male epithelial cells shed during intercourse may be a possible mode of transmission (155). It has been shown that certain probiotic lactobacilli are effective against the biofilm (109,133).

These findings suggest that different species are complexly interacting with each other (either in inhibition or in symbiosis) and this may determine whether the environment in the human vagina is healthy or not.

SEQUELAE AND COMPLICATIONS

Women having BV are at higher risk for acquiring Herpes simplex type 2 (22) and HIV (117) and different STIs, such as *C. trachomatis, N. gonorrhoeae, T. vaginalis* (2,175). The condition is also associated with pelvic inflammatory disease (66,153,174) and cervicitis (137,138).

Several studies have associated BV with second trimester pregnancy loss (63,98). BV is associated with amniotic fluid infection (143) and chorioamnionitis (70) and this may to some extent explain the relationship between BV and second trimester loss although the mechanisms are poorly understood and need further study.

Preterm birth is one of the most important issues in obstetrics

due to the high perinatal mortality and morbidity and there is a lot of literature about the relationship between this condition and abnormal vaginal microbiota. However, since it is not the main focus of this thesis, only a few selected studies will be presented here. Preterm birth has been associated with BV in some studies (63,71,108), while other studies did not find association between BV and spontaneous preterm birth (78,152,161). Larsson and colleagues found that treatment with clindamycin vaginal cream initiated at a mean gestational age of 96 days significantly prolonged the gestation in babies born preterm, but did not show any significant overall reduction of the rate of spontaneous preterm birth prior to 37 completed weeks (93). In Scandinavia the proportion of low birth weight and preterm births are among the lowest in the world. The discrepant associations between BV and preterm birth found in these studies may be explained by genetically determined variations in the immunological response to BV, both regarding the TNF alleles and interleukin production that could make women more or less susceptible to BV, causing different risks of preterm birth (91).

Presence of BV increases the risk of urinary tract infections both in non-pregnant (56) and pregnant women (65). Occasionally, urinary tract infections with BV-associated bacteria (i.e. *G. vaginalis*) are described (131,177), and they are more often reported in women than in men (18). Recently, *G. vaginalis* biofilm has been shown to be present on epithelial cells from urine both in women and in their male partners (154,158).

Thus, BV, being asymptomatic in about half of the women affected, could lead to serious complications both in non-pregnant and pregnant women and some of these complications may become life-threatening for the woman or her offspring.

TREATMENT

The recommended regimens for BV are metronidazole 500 mg orally twice daily for five to seven days, or metronidazole 2 g orally in a single dose, or clindamycin vaginal cream 2% once daily for 7 days, or clindamycin 300 mg orally twice daily for seven days (79,141) or metronidazole vaginal gel 0.75% once daily for five days or tinidazole 2 g orally in a single dose (32,141). None of the treatment modalities have been shown in controlled trials to be better than the others, although recurrence after single dose treatment appear to be slightly higher. Metronidazole and clindamycin are used in Denmark as tinidazole is not registered.

Most patients will experience recurrences of BV within 3-12 months whatever treatment has been used; adjuvant vaginal application of probiotics has been suggested and has shown some effectiveness in preventing the recurrences in a few studies (95,101) but is not generally recommended.

Although not reflected in most guidelines, pregnant high-risk women (i.e. women who have previously suffered pregnancy loss or preterm birth due to infection) who have abnormal vaginal flora should probably be treated with oral clindamycin early in pregnancy in order to prevent adverse pregnancy outcomes (89). In pregnancy, systemic therapy is generally the preferred route of administration, as it is speculated that a possible amniotic infection may be better treated. In non-pregnant women local therapy is as effective as systemic. In most studies, metronidazole has not prevented adverse pregnancy outcomes, but clindamycin has (88). Probably, however, this is also an effect of the timing of the treatment, as the clindamycin trials have been conducted much earlier in pregnancy, and it is considered likely that the inflammatory damage leading to preterm birth is an early event (89).

In vitro studies have shown that clindamycin has a better efficacy compared to metronidazole against *A. vaginae* (29,44), *G. vaginalis* (29) and *Mobiluncus* sp. (6). However, anaerobes such *Prevotella* spp. (both *P. bivia* and the black-pigmented *Prevotella* species) may become resistant to clindamycin (5).

For women undergoing some surgical procedures, i.e. total abdominal hysterectomy, treatment for BV can be initiated with metronidazole 1g rectally/ 500 mg orally the evening before surgery and after surgery for a minimum of 4 days, reducing significantly vaginal cuff infection (92).

BACTERIAL VAGINOSIS IN DENMARK

Relatively few studies have been performed in Danish women. Vejtorp at al. conducted a randomized controlled trial on the effect of metronidazole treatment of male partners on BV recurrence rate and showed that partner treatment did not affect recurrence rate (164).

Bro studied women in general practice, comparing microbial flora in women with and without vaginal discharge and showed that BV was present in 36% of women with discharge compared to 8% among those without (9) and later he showed that metronidazole pessaries were efficient in treating BV (10).

Petersen et al. surveyed BV in Danish women attending an STD clinic (124,125), while Schmidt and Hansen examined possibilities of diagnosing BV in Danish general practice (134,135). Thorsen and collaborators conducted a large study in nearly 3000 pregnant women and suggested a pathologic group of bacteria, consisting of *G. vaginalis*, anaerobes and *M. hominis* in pregnant women with BV (160). Later it was found that BV in early pregnancy was not associated with spontaneous preterm birth (161), while Svare *et al.* found that BV in the second trimester of pregnancy was a risk factor for indicated but not spontaneous preterm birth (152).

METHODOLOGY, PRINCIPAL FINDINGS AND CONCLUSIONS

General comments on methodology
Sample collection (self-taken versus physician-collected vaginal samples)
The women from Greenland included in Study I and Study III collected self-taken vaginal swabs, vaginal smears, and FVU. The women were given a pictorial instruction sheet about how to self-collect vaginal swabs by inserting a flocked swab approximately five cm into the vagina using the breaking score as a guide to ensure that the samples were collected properly.
Menard and colleagues showed that self-taken vaginal swabs were as reliable as practitioner-collected ones, as far as qPCR analyses for *Lactobacillus* species, *G. vaginalis* and *A. vaginae* were concerned (111). Bacterial vaginosis could be diagnosed by Nugent's criteria with self-taken vaginal smears and gynaecologist collected vaginal smears with a kappa value of 0.98 between these two sets of smears, suggesting a very high concordance (159). These studies supported the approach of self-taken vaginal swabs in study I and III.
An experienced physician collected samples from the vaginal fornix of the women included in Study II by using 10 µl plastic loops ensuring a uniform amount of sample, which may have provided more accurate estimates of the relevant cut-off for the species specific qPCRs; however, as both the self-taken and physician collected specimens generally gave comparable sensitivities

and specificities, the difference appears to be of minor importance.

Transport medium

Women included in Study I and III provided vaginal swabs specimens collected in Copan UTM (Copan, Brescia, Italy) and FVU collected in GeneLockTM (Sierra Molecular Corp., Sonora, CA, USA). All the biological samples were stored in a refrigerator at 5ºC until they were sent at ambient temperature to Statens Serum Institut in Denmark. Samples were sent in weekly shipments from Nuuk and in one bulk shipment from Sisimiut to Statens Serum Institut. Both the UTM and the GeneLock transport medium stop the growth of bacteria in the sample, although the GeneLock medium also provide protection against nucleic acid degradation for extended periods of time, even at room temperature. However, comparing the results of Study I and III suggests that the DNA degradations must have been minimal, as shown by the good correlation between bacterial load in swabs and urine.

In Study II vaginal secretions were placed in phosphate-buffered saline with 10% glycerol and frozen immediately at -70ºC, thus ensuring the optimal preservation of the specimens. This approach was possible as the samples were collected in a university hospital setting, in contrast to the field conditions in Greenland.

DNA extraction methods, advantages and limitations

The extraction method used for samples from Study I and III was a simple lysis procedure with boiling of the sample in the presence of Chelex 100 (BioRad, Hercules, CA, USA) in TE buffer. This method has been used for years as the standard procedure for all clinical specimens examined in the diagnostic PCR assays for various STIs and has been found to be highly efficient and robust. However, due to concern that Gram positive species, in particular lactobacilli, would be incompletely lysed by this procedure, a series of experiments were conducted to evaluate this. As the *L. gasseri* qPCR was the first assay among the four qPCRs for lactobacilli, initial studies were performed with this species.

Experiment 1

From the diagnostic laboratory, 18 vaginal swab specimens were selected and DNA was extracted by the standard Chelex procedure (50 µl sample mixed with 150 µl 20% Chelex 100 in TE buffer) and by QIAamp extraction with a lysis buffer and mutanolysin protocol previously published (28). Negative controls (PCR-grade water) were extracted by both methods. The samples were tested in duplicate for *L. gasseri* together with four extraction controls. Unfortunately, the negative controls for the QIAmp method contained approximately 15 copies of *L. gasseri* DNA, however, the results could be interpreted as 9 samples extracted by the standard Chelex protocol were negative but became positive when extracted by De Backer's method and 5 of them contained more than 15 DNA copies of *L. gasseri*. An additional 5 samples were positive when extracted by the standard Chelex protocol but contained between 7 to 40 times more DNA copies when extracted by the De Backer's protocol. Only 4 samples remained negative by both extraction methods. *L. gasseri* was analysed in a SYBR-Green qPCR assay using an ABI 7500 instrument with the following cycling conditions: an initial 10 min denaturation followed by 10 cycles of touch-down PCR with 1ºC decrement per cycle from 70ºC until reaching the annealing temperature of 60ºC, which was used for the subsequent 30 cycles followed by a melt-curve analysis.

Experiment 2

A total of 27 vaginal swabs from the diagnostic laboratory were extracted using the standard Chelex procedure and a modified QIAamp Mini DNA Kit (Qiagen, Hilden, Germany) protocol where 200µl of the sample was mixed with 400 µl of lysis buffer (20mM Tris-HCl, pH 8; 2mM EDTA; 1.2% Triton) and 6 mg lysozyme in 15 µl TE was added and incubated at 37ºC for 30 min. This protocol is similar to that published by De Backer et al. (28) with the exception that lysozyme was used instead of mutanolysin. The subsequent steps were carried out according to the QIAamp standard procedure.

Results from qPCR in duplicate for *L. crispatus*, *L. gasseri*, *L. jensenii* and *L. iners* were compared and presented in the figures below where the mean number of copies is plotted as log10 of the absolute copy number plus one, so the negative samples are shown as 0 on the log scale.

Figure 18:
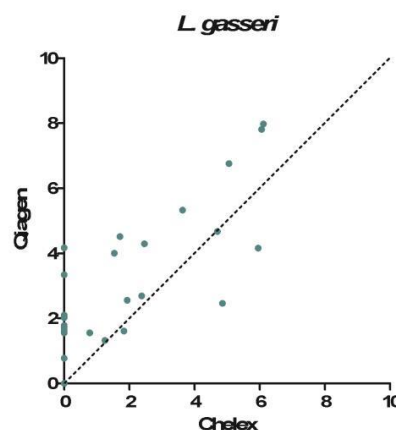
Chelex versus modified QIAamp extraction on 27 samples for L. gasseri. The dotted line on the graph has the slope = 45º.
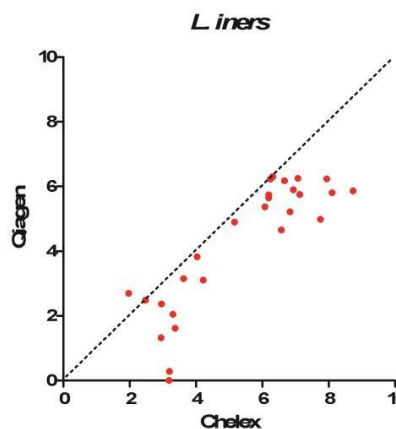
Figure 19:

Chelex versus modified QIAamp extraction on 27 samples for L. iners. The dotted line on the graph has the slope = 45º.

Figure 20:
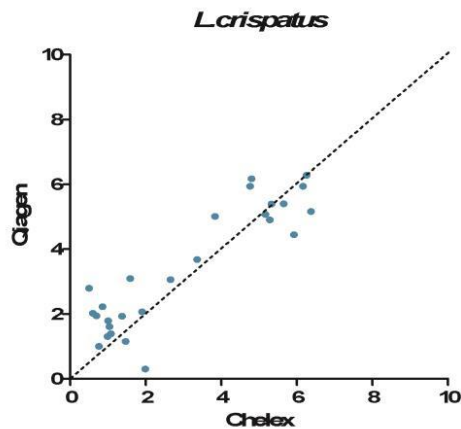
L.crispatus

Chelex versus modified QIAamp extraction on 27 samples for L. crispatus. The dotted line on the graph has the slope = 45º.

Figure 21:
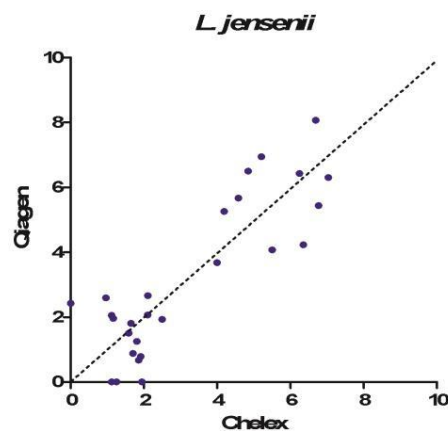
L. jensenii

Chelex versus modified QIAamp extraction on 27 samples for L. jensenii. The dotted line on the graph has the slope = 45º.

Mann-Whitney U test showed that the modified QIAamp and the Chelex standard protocol performed similarly for *L. crispatus* and *L. jensenii* with p-values 0.39 and 0.96, while for *L. gasseri*, the modified QIAamp extraction was significantly better than Chelex (p=0.015). In contrast, the Chelex protocol was significantly better than modified QIAamp for *L. iners* (p=0.016). Consequently, only *L. iners* PCR was performed on vaginal swabs from Study I. It could have been relevant to include both the *L. crispatus* and *L. jensenii* qPCRs for the vaginal samples but as these species are uncommon within BV communities, the results for Study I are not affected by the lack of qPCR for these lactobacilli.

The DNA extraction method for samples from Study II performed on 100 µl of vaginal sample with the DNeasy blood and tissue kit (Qiagen) including a proteinase K incubation according to the manufacturer's instructions. Final elution was with 100 µl (2x50 µl) of elution buffer.

qPCR (SYBR-Green versus TaqMan)
BV associated bacteria were quantified by 11 qPCRs (BVAB 1, 2, 3, TM7, *Megasphaera* type 1 and 2, *Eggerthella*-like bacterium, *M. curtisii* and *M. mulieris*, *Prevotella* spp. and *F. magna*) assays,

which were Sybr-Green based and 8 qPCR assays that were Taq-Man based.

SYBR Green dye binds to the double stranded DNA and fluoresces 1000 times brighter when bound than when unbound. The SYBR Green signal increases as the PCR generates more double-stranded product. The disadvantage of the SYBR Green system is that it detects all double stranded DNA. Primer dimers and non-specific PCR products will give a signal identical to that of the real product. In order to overcome this problem, a melting curve was performed to determine the composition of the reaction product and extra emphasis was put on proper primer design and PCR optimization.

In a TaqMan system, a probe specific for the target gene is added to the PCR mix. The TaqMan probe binds to the single-stranded DNA at a combined annealing and elongation step. Attached to the probe are a reporter dye and a quencher dye, which is sepa-rated from the reporter due to the 5'exonuclease activity of the Taq polymerase resulting in the emission from the reporter dye in a positive reaction. The probe provides the TaqMan assay with a higher specificity and in most assays also a lower limit of detec-tion. Furthermore, multiplexing is possible as different reporter dyes can be used. The disadvantage of this system is that the cost of the probes makes it more expensive than the SYBR Green assay and that it is more time consuming since a probe must be de-signed.

An internal amplification control was developed for 6 of qPCR TaqMan assays (*A. vaginae*, *L. amnionii*, *S. sanguinegens*, *M. hominis*, *U. parvum* and *U. urealyticum*) in order to detect the presence of PCR inhibitors in the samples and thus, to avoid false negative results. Two qPCR were TaqMan but without internal control (*L. iners* and *G. vaginalis*). In order to estimate the robust-ness and the influence of non-target DNA on the SYBR-green assays (BVAB1, 2, 3, TM7, *Eggerthella*-like bacterium, *Megasphaera* type 1 and 2, *M. curtisii*, *M. mulieris*, *Prevotella*) and *G. vaginalis* TaqMan assay, the standard-curves were evalu-ated with and without the presence of negative clinical samples. All four real-time PCR for STIs were TaqMan assays with internal control for inhibition but the results were expressed as posi-tive/negative and positive results were confirmed by another PCR target from the organism in order to ensure an optimal specificity as previously described (54).

Standards (rRNA gene copies)
For the uncultured bacteria BVAB 1, 2, 3, TM7, *Megasphaera* type 1 and 2, and *Eggerthella*-like bacterium, the PCR product from a positive clinical sample was used as positive control after gel-purification with a QIAquick Gel Extraction Kit (Qiagen) and DNA sequencing for verification. Positive controls for the PCRs were obtained by DNA extraction from cultures for the bacteria, for which culture was possible.

Genomic and amplified DNA for positive controls was quantified fluorometrically with a Qubit fluorometer, using the Quant-iTTM dsDNA HS Assay Kit (Life Technologies Corp., Paisly, UK). Standard curves for quantitative PCRs were generated using 10-fold dilu-tions from 1 genome equivalent (geq) /µl to 10^7 geq /µl in TE buffer containing 1 µg/ml of calf thymus DNA (D-8661; Sigma-Aldrich).

All results were expressed as number of 16S rRNA gene copies/ml UTM for Study I and III and 16S rRNA gene copies/µl of extracted DNA for Study II.

It should be noted that the number of rRNA operons is unknown for most of the uncultured bacteria and as PCR products are used,

the results reflect the number of 16S rRNA-gene copies whereas for cultured bacteria, genome equivalents were calculated from the genome size of the organism in relation to the concentration of genomic DNA. However, as most bacteria have 1-5 16S operons within the genome, and since the results were comparable within the assay, the inaccuracy in quantity was deemed to be unimportant.

454-pyrosequencing
Pyrosequencing of PCR amplicons produced with universal 16S primers provide a culture-independent image of the microbiota. Because pyrosequencing is a single-molecule technique, the 16S rDNA PCR products can be characterized directly without cloning, saving a lot of work and being also more affordable than traditional methods such as Sanger sequencing (97). Several thousand sequences can be obtained per sample thereby enabling a quantitative description of a complex microbiota.

A limitation of pyrosequencing is the short read lengths, providing sometimes only a genus-level assignment and a less sensitive assay compared to PCR performed with specific primers. Furthermore, the formation of chimeras may lead to an overestimation of the species diversity. Some of these disadvantages are discussed in Study II, where pyrosequencing results are compared with qPCR results.

BV scoring (Nugent versus Amsel versus Claeys)
The most used microscopic method for diagnosing BV is the Nugent score (120). It provides a quantitative and therefore more objective and reproducible method of evaluating vaginal flora, but it has been discussed that this method leaves several BV-associated bacterial morphotypes unidentified or that bacteria like *Mobiluncus* spp. may be overrepresented due to the similar morphology and staining characteristics of BVAB 1 (90), with curved rod morphology demonstrated by FISH (48). It has also been suggested that the Nugent score is subjective, tending to over-diagnose BV and the large proportion of asymptomatic women with BV on Nugent scores is a problem (99). Donders has mentioned that the method applied to vaginal smears prepared from Stuart's transport medium stored for 3-6 hours underestimates the number of lactobacilli in grade I flora when compared to fresh Gram stained smears leading to a misclassification into grade II (39). It should be noted, however, that this study applied fixation with alcohol which may be less efficient than heat fixation and particularly for smears prepared from Stuart's medium as these may contain more moisture and may be difficult to dry completely.

Vaginal smears collected within Study I were fresh, directly applied on a glass and heat-fixed; they were also evaluated by using Claeys' criteria. This was done 4 weeks after Nugent evaluation and was blinded to any previous clinical or laboratory results. Table 1 shows the correspondence between Nugent's and Claeys' systems.

Nugent's classification has been shown to be in good agreement with Amsel's and Ison-Hay's criteria and with wet-mount microscopy (47). The reproducibility of BV diagnosis by using Nugent and Ison-Hay was indirectly confirmed during the present PhD study, as there was a good agreement between Nugent's and Claeys' scoring systems with a Cohen's kappa of 0.90, and as the latter is based on Ison-Hay's criteria, but more detailed (166).

A debated area is represented by Nugent's intermediate flora, and further detailed analyses were performed on the samples from Study I where discrepancy was found between Nugent and Claeys. Two smears classified as Nugent II were scored 1-like in Claeys' classification, both with bacterial loads for *L. amnionii* and *Prevotella* spp. over cut-off when compared to the eight participants with 1-like in agreement with Nugent grade I. Eleven participants were diagnosed with Nugent grade II and Claeys grade 3 and they had *A. vaginae* and *G. vaginalis* at significantly lower concentrations than the 79 women with Nugent III and Claeys 3. It remains controversial how to handle intermediate flora (35). Intermediate flora has been described as a less distinct transitional pattern between normal flora and BV (69) and pregnant women with BV-like flora (either grade II or III) or grade I-like or grade I-PMN diagnosed once during pregnancy either in first or second trimester, seem to be more prone to adverse pregnancy outcome such as preterm birth, with the odds-ratios for preterm birth associated with BV-like flora being less than odds-ratios for preterm birth associated with grade I-like or I-PMN (171). Whether molecular diagnosis would allow for a more accurate classification remains to be evaluated.

We found a high prevalence of BV in the Greenlandic population of 45 % and 51 % as diagnosed by Nugent's and Claeys' scores, respectively. A possible explanation for this high prevalence might be the composition of normal flora as determined by Claeys' score with grade 1a, consisting of *L. crispatus* and *L. jensenii* (166), found in only 3 % and with grade 1b, consisting of *L. iners* and *L. gasseri*, (166) found in 13 %, as the latter was associated with a lower stability of the flora, i.e. a type of flora more prone to shifting towards BV (169).

Table 1:

Nugent		Claeys	
Grade	[1]N (%)	Grade	[1]N (%)
I	73 (41)	1a	6 (3)
		1b	22 (12)
		1 ab	34 (19)
		1-like	8 (4)
		2	3 (2)
II	25 (14)	1b	1 (1)
		1 ab	1 (1)
		1-like	2 (1)
		2	5 (3)
		3	11 (6)
		4	5 (3)
III	79 (45)	3	79 (45)

Correlation between Nugent's and Claeys' criteria.

[1]N indicates number of participants (total 177 women).
I indicate normal (score 0-3), II intermediate (score 4-6) and III BV flora (score 7-10) as determined by Nugent's criteria.
1a indicate normal flora (as determined by Ison-Hay) with thick and short lactobacilli, as detailed by Claeys.
1b indicate normal flora (as determined by Ison-Hay) with thin and long lactobacilli, as detailed by Claeys.
1ab indicate normal flora (as determined by Ison-Hay) with a combination of thick, short and thin, long lactobacilli, as detailed by Claeys.
2 indicate intermediate flora as determined by Ison-Hay.
3 indicate BV as determined by Ison-Hay.
1-like indicate a separate category defined neither by Nugent nor Ison-Hay, consisting of small, irregular shaped Gram positive rods with a disposition similar to Chinese letters.

Quantitative detection (Use of ROC curve analysis)
As most of BV associated bacteria are present both in healthy women and women with BV, we introduced threshold quantification in order to optimize the diagnostic accuracy of the molecular techniques by using Receiver Operating Characteristic (ROC) to define cut-off levels for diagnostic tests. Quantitative detection considers samples with a gene copy number higher than an established value (referred to as threshold or cut-off) as positive, and samples with bacterial loads below this threshold as negative. In qualitative detection only presence vs. absence is considered. The sensitivity and specificity of the assays was determined both for qualitative and quantitative detection using Nugent's score (grade I vs. grade III) as reference (Study I and III) and Amsel's criteria (normal versus BV) (Study II). Samples with intermediate flora (grade II) by Nugent (Study I and III) and partial BV as determined by wet-mount (Study II) were excluded from this analysis.

STUDY I
Vaginal microbiome in women from Greenland assessed by microscopy and quantitative PCR
BMC Infect Dis 2013 Oct 16; 13 (1):480.

Methodology
Microscopy of self-collected vaginal smears from 177 women in Greenland was performed according to Nugent's criteria. Bacterial cells were lysed using boiling with Chelex 100. Four PCRs for sexually transmitted infections (STIs) and 19 qPCRs for different vaginal bacteria were performed on vaginal swabs from these women. Receiver Operating Characteristic (ROC) curve analysis was applied, using Nugent's score as gold standard for BV. Cut-offs values for optimal BV prediction were determined, as well as sensitivities (Se) and specificities (Sp) in qualitative and quantitative detections. Associations between different vaginal bacteria and BV were evaluated both in univariate and multivariate analyses. A heat-map with dendrogram was created and clustering/sub-clustering of the 19 vaginal bacteria was shown, also in relation to Nugent's classification. Co-occurrence of bacterial species was shown by constructing a heat-map of hierarchically clustered Spearman correlation coefficients between bacterial species.

Principal findings
BV was highly prevalent in these women with 45% BV according to Nugent's score. STIs were common, but there was no statistically significant relationship between each STIs taken separately and BV or between the lifetime number of male sexual partners and BV. However, when considering either *M. genitalium* or *C. trachomatis* or *T. vaginalis* or *N. gonorrhoeae,* infected women more often had BV than normal flora (p = 0.04).
Seven vaginal bacteria with largest areas under curve (AUCs in brackets) after applying ROC curve analysis were selected: *A. vaginae* (97%), *Prevotella* spp. (96%), *G. vaginalis* (95%), BVAB2 (94%), *Eggerthella*-like bacterium (91%), *L. amnionii* (89%), and *Megasphaera* type 1 (88%). These seven bacteria were analysed in a multivariate logistic regression model, and two of them, *Prevotella* spp. (p = 0.0095) and *A. vaginae* (p = 0.0326) remained associated with BV after adjusting for the others. Only three species (*U. parvum, U. urealyticum* and *L. iners*) were not associated with BV in univariate analysis and quantitative detection.

Among women with BV, three clusters were dominated by single taxons, i.e. *Prevotella* spp., BVAB1, *G. vaginalis*, one cluster was dominated by *G. vaginalis/ Prevotella* spp. and another by BVAB1/ *G. vaginalis*. The rest of the BV clusters had multiple bacterial species. On a correlation heat-map the most significant cluster of correlations was found between BVAB 2 and *A. vaginae, S. sanguinegens, L. amnionii,* BVAB 3, *Megasphaera* type 1 and *Eggerthella*-like bacterium.
Using quantitative detection, Nugent as gold standard and combining "*Prevotella* spp. and/or *A. vaginae*" PCR results, it was possible to select either an almost perfect specificity or an optimal sensitivity for diagnosing BV.

Conclusions
This study characterises for the first time in detail the vaginal flora in a cohort of women from Greenland using microscopy and qPCR. A high prevalence of BV was found in this population. PCR can be used as an accurate tool of diagnosing BV and the best combination was found between "*Prevotella* spp. and/or *A. vaginae*". Further, different clusters of BV could be identified in this population, some dominated by one species, others by species in pairs, suggesting metabolic co-dependencies between these. Stratifying BV would possibly allow future targeted treatment modalities.

STUDY II
Composition of the vaginal microbiota in women of reproductive age – sensitive and specific molecular diagnosis of bacterial vaginosis is possible?
PLoS One 2013 Apr 9; 8(4):e60670.

Methodology
BV and normal flora was diagnosed by Amsel's criteria on a cohort of 163 Swedish women. Intermediate cases were diagnosed using wet mount microscopy according to Donders' Lactobacillary grade classification as Lactobacillary grade II, which corresponds to partial BV and should not be confused with the intermediate category as determined by Nugent score. DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen). Vaginal samples were analysed using 454 pyrosequencing of the hypervariable region V4 of the 16S rRNA gene and 16 qPCRs identical to those used in Study I with the exception of the *Prevotella* PCR, which was less broadly reactive, including only 3 species in contrast to that from Study I, which captured 20 species. Quantitative results were expressed as relative abundance for 454 sequences and optimal thresholds for qPCR results, both as determined by ROC curve analysis, using Amsel criteria for normal and BV flora as gold standard. Agreement between the presence of an individual species/ genus, as determined by 454 pyrosequencing versus real-time PCR was measured by calculating kappa values.

Principal findings
The same seven bacteria as in Study I (*A. vaginae, G. vaginalis*, *Eggerthella*-like bacterium, *Megasphaera* type 1, *Prevotella*, BVAB2, *L. amnionii*) showed the largest AUCs for qPCR, with *A. vaginae* having the highest diagnostic accuracy. When ROC curve analysis was applied for the relative abundances of the bacteria as measured by 454 pyrosequencing, the best AUCs (> 0.90) were obtained for *A. vaginae, Prevotella*, *Eggerthella* and *Megasphaera* type 1, while *G. vaginalis*, BVAB 2 and *L. amnionii* had AUCs > 0.85. The highest sensitivities and specificities were obtained

when the depletion of *Lactobacillus* spp. (relative abundance ≤ 47 %) was combined with the presence of either *G. vaginalis* or *A. vaginae* (after ROC determined threshold) by qPCR (Se = 97 %, Sp = 96 %) or by 454 pyrosequencing (Se = 100 %, Sp = 95 %). Kappa values indicated fair to good agreement for the majority of species / genus as determined by 454 pyrosequencing and qPCR. Most cases of disagreement were samples negative by 454 sequencing and positive by qPCR, which was expected as the latter method is more sensitive. For the majority of discrepant cases the amount (quantities for qPCR and abundance for 454 sequencing) of bacteria was very low, and under the diagnostic thresholds determined by ROC curve analysis.

Conclusions

This paper confirms the results from Study I by finding the same seven bacteria highly predictive for BV with the best diagnostic accuracy shown for *A. vaginae*. The depletion of *Lactobacillus* spp. as determined by 454 pyrosequencing combined with the presence of either *G. vaginalis* or *A. vaginae* at diagnostic levels, as determined either by quantitative qPCR or 454 pyrosequencing, was a precise BV predictor. Fair to good agreement was found between the presence of an individual species/genus by 454 pyrosequencing and qPCR. Before applying these approaches in clinical practice, the results should be validated on other populations in order to prove their generalizability. However, the time and effort needed to interpret the 454 data will probably limit the usefulness of this method outside of research settings.

STUDY III
Bacterial vaginosis diagnosed from first void urine specimens
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Methodology

The same population as included in Study I was used. Results of classification by microscopy of the 176 vaginal smears by Nugent's criteria were used from Study I. DNA from the FVU samples was extracted using the Chelex 100 method similar to the DNA extraction from swabs. qPCRs for seven selected vaginal bacteria (*A. vaginae*, *Prevotella* spp., *G. vaginalis*, BVAB 2, *Eggerthella*-like bacterium, *L. amnionii, Megasphaera* type 1) were performed on the FVU samples, as they showed a good prediction for BV in swab samples having AUCs > 85 % (Study I). ROC curve analysis was applied using Nugent's score as gold standard, and cut-off values for optimal BV prediction from FVU samples were determined, as well as sensitivities and specificities for BV diagnosis from FVU samples in qualitative and quantitative detection. Spearman correlation coefficients were determined between the seven species found in swabs and FVU, as well as between these species from FVU. Agreement between BV as diagnosed by Nugent score and qPCR on FVU and swab samples were measured using Cohen's kappa. Associations between different bacteria determined in FVU samples and BV were evaluated both in univariate and multivariate analyses.

Principal findings

The seven bacterial species showed almost the same AUCs as in swab samples (Study I), when ROC curve analysis on FVU samples was performed using Nugent score as gold standard. When comparing specificities and sensitivities for molecular BV diagnosis from swabs and FVUs, no statistically significant difference was found.

In order to estimate how well bacteria from swabs and FVU samples correlated, Spearman correlation coefficients were calculated and the highest was found for *A. vaginae*, while the lowest was for *Prevotella* spp. The correlation was compared using regression lines for each species after log_{10} transformation of the bacterial load with vaginal bacterial load as the predictor. The seven slopes were compared two by two and the slope for *Prevotella* spp. was statistically significantly different from the slopes of the other six bacteria. Intercepts were between [-0.45; 0.52] except for *Prevotella* spp., which had an intercept of 2.42 indicating that this species could be found in higher quantities in urine without being present in swabs.

Co-occurrence of bacterial taxa in urine was also investigated by calculating Spearman correlation coefficients with the best positive correlation (0.88) found between *A. vaginae* and *G. vaginalis*. A good and very good agreement was found between Nugent and molecular diagnosis of BV from FVU samples and swabs, and also between FVU and swabs with Cohen's kappa between 0.74 and 0.91.

Each of the seven bacteria detected in FVU samples were significantly associated with BV in univariate analysis before applying cut-offs and the association became stronger when quantitative detection was used. In a multivariate analysis, only *Megasphaera* type 1 and *Prevotella* spp. remained associated with BV after adjusting for the other five bacteria (p = 0.0167 and p = 0.0409, respectively).

Conclusions

This study is the first to investigate the possibility of diagnosing BV from FVU by qPCR. A combination of *Megasphaera* type 1 and/or *Prevotella* spp. was found to diagnose BV with the highest accuracy. Diagnosing BV from FVU was not statistically different from diagnosing BV from swabs. For culture negative women with persisting UTI symptoms the possibility that vaginal bacteria may cause urinary tract infections is worth investigating, and PCR would represent an important diagnostic tool as many of these bacteria cannot be cultured.

DISCUSSION OF THE RESULTS

Molecular methods for diagnosing BV
Molecular methods performed on swabs (Study I and II)
Verstraelen revealed in 2004 by culture-independent methods the association between *A. vaginae* and BV (170). Sha *et al*. was the first group to investigate the use of qPCR for the diagnosis of BV, studying *Gardnerella vaginalis*, *Mycoplasma hominis* and *Lactobacillus* spp. in HIV positive women (140). Since 2005 when Fredricks *et al*. reported on the finding of new uncultured bacteria associated with BV (48), the BV literature has exploded with newly developed molecular methods for detecting and quantifying these new bacteria and for diagnosing BV.
Using previously published primers (28,49,50,179), some of them slightly modified in 5'-end, we established 16 real-time PCR assays for BV associated bacteria. These were used both in Study I and II in the same manner with the exception of the *Prevotella* PCR assay, which in Study II covered only three species. This was for Study I changed to cover a broader spectrum of species (now matching 20 different *Prevotella* spp.). Besides, three qPCR for *U. parvum, U. urealyticum* and *M. hominis* and four PCR assays for STIs were further used in Study I.

The same seven BV-associated bacteria were found to have the best diagnostic accuracy when applying ROC curve analysis for qPCR results (AUC in brackets, respectively for Study I and II): *A. vaginae* (97% and 99 %), *Prevotella* spp. (96% and 94 %), *G. vaginalis* (95% and 96 %), BVAB2 (94% and 93 %), *Eggerthella*-like bacterium (91% and 96 %), *L. amnionii* (89% and 89 %), and *Megasphaera* type 1 (88% and 96 %). Some of these species have earlier been reported to predict BV accurately (17,50,110). These similarities are encouraging as they indicate that the findings may be generalizable across populations with different ethnic composition.

A. vaginae was found to have the largest AUC in both studies, and therefore the highest diagnostic precision. Notable differences in AUCs between Study I and II could be noted only for *Eggerthella*-like bacterium and *Megasphaera* type 1, but the difference was statistically significant only for the latter. This difference can be explained by the fact that different gold standards for diagnosing BV were used (Nugent's score for Study I versus Amsel criteria for Study II) and by differences in the populations (Greenlandic and Swedish populations included in Study I and II, respectively), as it is known that race and different practices, that are common in certain geographical areas are decisive for vaginal health. The median age of the participants in the two selected populations was the same (24 years) for Study I and II, but due to the design and primary aims of Study I, more elderly women (between 55-65 years) were included. These women are presumably postmenopausal and their hormonal status certainly affects the vaginal microbiome. Data were not recorded about recent antibiotic use and contraceptive use for women in Study I and these may be potential confounders. Study II included women between 15-54 years and all were in reproductive age, and thus it was a more homogenous population. The thresholds for bacterial loads calculated in Study I and II were not comparable. This can be explained by the different DNA extraction methods that were used (Chelex and Qiagen, respectively), different methods of collecting the vaginal samples: self-collected by inserting a flocked swab about 5 cm into the vagina or collected by a physician from the vaginal fornix using 10 μl plastic loops, as well as by different methods used for diagnosing BV.

Molecular methods performed on first void urine (Study III)

In Study III, 176 FVU samples were analysed for the seven vaginal bacteria, which had the best AUCs after applying ROC for qPCR data in Study I and II. We found no statistically significant differences in sensitivities / specificities for diagnosing BV by qPCR between swabs and urines, indicating that FVU is a reliable sample for molecular diagnosis of BV. Besides, by combining *Megasphaera* type 1or *Prevotella* spp., the sensitivity reached 99 % and the specificity was maintained at 95 %; *A. vaginae* or *Megasphaera* type 1 or *Prevotella* spp. resulted in a sensitivity of 99 % with a specificity of 93 %, while *A. vaginae* or *Megasphaera* type 1 or *Eggerthella* provided a sensitivity of 96 % and a specificity of 97 %, all by quantitative detection.

A good or very good agreement was found between Nugent score and molecular diagnosis of BV from swabs and FVU samples and between swabs and FVU samples using Cohen's kappa.

These findings indicate that urine can be used to diagnose BV. This is useful in studies based on urine sampling, when BV was not considered initially.

Vaginal flora divided into clusters and sub-clusters of bacteria (Study I)

The women with BV from Study I could be further divided into clusters and sub-clusters of BV dominated by *Prevotella* spp., BVAB1 and *G. vaginalis* as single bacterial species or by combinations of bacteria such as *G. vaginalis* with *Prevotella* and BVAB1 with *G. vaginalis*. This approach has also been used in other populations with species quantitation based on 454 pyrosequencing of amplicons (151) and our findings are similar to these, except for a *Sneathia/Leptotrichia* spp. dominated cluster which was not prominent in Study I. These differences could be due to differences in the methods used (454 pyrosequencing versus species/genus-specific qPCRs) or due to real differences between populations.

The associations between bacteria within the BV population could be explained by symbiotic relationships between bacteria, i.e. *Prevotella-G. vaginalis* (128) or by synergistic actions, as it has been suggested that *G. vaginalis* is the most pathogenic organism, creating biofilm on which other BV-associated bacteria would attach (156,168).

Dividing BV into clusters/sub-clusters of bacteria could have important implications for the treatment of the condition, which can be targeted according to the relevant subgroups, as some BV associated bacteria have different resistance patterns. This may be particularly important in pregnant women with BV, where a tool for differentiating high-risk from low-risk BV would be extremely important in order to limit unneeded use of antibiotics.

Comparison of BV-associated bacteria from swabs and first-void urine (Study I and III)

All seven selected species, *A. vaginae, Prevotella* spp., *G. vaginalis*, BVBA2*, Eggerthella*-like bacterium, *L. amnionii* and *Megasphaera* type 1, both from swabs and FVU samples were significantly associated with BV in univariate analysis and quantitative detection. The highest odds-ratios were found for *Prevotella* spp. in swabs (OR = 437) and *A. vaginae* from FVU samples (OR = 730).

In multivariate logistic regression analyses, *Megasphaera* type 1 and *Prevotella* spp. from FVU remained associated with BV, while *A. vaginae* and *Prevotella* spp. from swabs remained associated with BV, after adjusting for the other five bacteria. The reason for this difference could not be explained.

The relation between the bacterial loads in swabs and in FVU samples could be modelled by performing linear regression analyses and comparing regression lines for each studied species (Study III). The DNA load detected in urines may represent only contamination from vagina; however, the idea that urinary tract infection could be caused by BV-associated bacteria should not be ignored and is well worth investigating. It has been reported that UTIs may be caused by BV bacteria, for example *G. vaginalis* (18) or *Prevotella* (11).

Prevotella spp. could be present in urine in high numbers even in women with vaginal smears diagnosed as Nugent I and II (overall intercept = 2.42); however, *Prevotella* loads in urine increased with Nugent's grades of vaginal smears, having highest load in women with BV. As urine is not routinely cultured anaerobically, the high load of several BV associated organisms merit further investigation. It is important to note, however, that in the present study, FVU was analysed, whereas mid-stream urine should be analysed for diagnosis of UVI.

CONCLUSIONS AND PERSPECTIVES FOR FURTHER RESEARCH

Conclusions of the PhD thesis

BV can be diagnosed by molecular methods performed either on swabs or urine but it is important to apply thresholds in order to improve the accuracy of the diagnosis. The presence of the same bacteria (*A. vaginae, Prevotella* spp., *G. vaginalis*, BVBA2, *Eggerthella*-like bacterium, *L. amnionii* and *Megasphaera* type 1) having the largest AUCs among two different populations (Greenlandic and Swedish) suggest that the findings are generalizable, although further validation of the results on other patients groups and in other geographic settings is required before using qPCR as a routine diagnostic tool. The molecular diagnostic methods were compared both with classical microscopic and clinical grading systems of diagnosing BV showing good agreement. This represents another way of demonstrating their reliability. It is necessary to evaluate both the generalizability and reliability of these methods as they are more expensive than classical methods (Amsel, Nugent, wet-smear microscopy). However, they can provide additional information, as they may allow evaluating the association between individual BV associated bacteria and BV complications such as preterm birth, cervicitis, miscarriage and PID.

It was shown that it was possible to identify clusters of BV dominated by single or paired bacteria, and these clusters could classify BV into subgroups, providing a more detailed understanding of the condition. This may allow for a more differentiated treatment and risk prediction in the future.

Finally, the possibility of urinary tract infections caused by BV-associated bacteria was suggested, and the molecular methods developed within this PhD project may be important tool for future studies, as most of the BV associated bacteria are not easily cultivable.

Perspectives for further research

The value of the qPCRs for diagnosing BV as well as the cut-off levels determined by ROC curve analysis need to be validated on other populations in order to generalize the findings. In order to do so, vaginal swabs submitted to Statens Serum Institut for BV diagnosis from 200 Danish women have been collected. The seven selected qPCR will be performed and results of Gram stained vaginal smears (prepared from the transport medium) will be compared.

An initial purpose of the present PhD study was to investigate adverse pregnancy outcomes in women with and without BV using microscopy and qPCRs. Unfortunately, the collected vaginal swabs and urines were not suitable for qPCR, as the bacterial DNA had deteriorated under storage. Future studies using fresh samples should, however, definitely be performed in order to evaluate whether the stratification of BV according to the dominant flora will improve the prediction of adverse pregnancy outcome. Such future studies should preferably involve sampling as early as possible in pregnancy.

Future studies should also address the role of BV associated bacteria in urinary tract infection. Such studies should include pregnant women as UTI during pregnancy is a known risk factor for adverse pregnancy outcome. If BV associated bacteria are associated with UTI, the detailed knowledge about the species

distribution provided by the qPCR methods may prove to be valuable in targeting the treatment according to aetiology.

Most studies of vaginal microbiology and also the present PhD thesis have employed cross-sectional study designs, in which the samples are obtained from participants at a single time point or with long intervals (weeks or months) between sampling. By this approach fluctuations in the vaginal flora, such as those caused by the menstrual cycle are missed. Longitudinal studies with frequent sampling are needed in order to study the dynamics of the vaginal flora.

CONFLICTS OF INTERESTS

I have no conflicts of interest.

SUMMARY

Background

Bacterial vaginosis (BV) is an imbalance of the vaginal bacterial microbiota and its aetiology is still unknown. Our aims were to investigate the diagnostic potential of species/genus specific quantitative PCR (qPCR) for bacteria present in swabs and first-void urine (FVU) samples using Nugent's and Claeys' criteria and 454 sequencing of the vaginal microbiome as reference.

Methods

Self-collected swabs, vaginal smears and FVU were obtained from 177 women from Greenland (Study I and III) and physician-collected vaginal swabs and smears were obtained from 163 Swedish women (Study II). BV was diagnosed by Nugent's criteria in Study I and III and by Amsel's criteria in Study II. The vaginal swabs and FVU samples were analysed by qPCR for selected vaginal bacteria in all three studies and for four sexually transmitted infections (STIs) in Study I.

Results

Study I: STIs were common in women from Greenland and BV was found in 45 % of these women but was not associated with individual STIs. In multivariate logistic analysis, *Atopobium vaginae* and *Prevotella* spp. were both independently associated with BV in swabs. BV could be subdivided into clusters dominated by a single or a few species together. Seven vaginal bacteria (*A. vaginae, Prevotella* spp. *Gardnerella vaginalis*, Bacterial vaginosis associated bacterium (BVAB) 2, *Eggerthella*-like bacterium, *Leptotrichia amnionii* and *Megasphaera* type 1) had areas under the receiver operating characteristic (ROC) curve > 85% in swabs, suggesting that they were good predictors of BV according to Nugent.

Study II: For the majority of species/ genera, the kappa values indicated fair to good agreement when their presence was determined by 454 pyrosequencing versus real-time PCR. The same seven vaginal bacteria as found in Study I, had areas under the receiver operating characteristic (ROC) curve > 85% in swabs from Swedish women, demonstrating a good diagnostic accuracy for BV according to Amsel.

Study III: In a multivariate model, *Megasphaera* type 1 and *Prevotella* spp. remained significantly associated with BV in FVU samples. A linear regression analysis showed good agreement between bacterial load from swabs and FVU, but *Prevotella* spp.

could be detected in high numbers in a few FVU samples without being present in swabs. After applying ROC curve analysis, the same seven vaginal bacteria as previously mentioned showed good prediction for BV according to Nugent in FVU. BV could be detected with comparable sensitivity in FVU and vaginal swabs.

Conclusions
BV can be diagnosed by molecular methods performed either on swabs or urine but it is important to apply thresholds in order to improve the accuracy of the diagnosis.
Further it was possible to identify clusters of BV dominated by single or paired bacteria, and these clusters could classify BV into subgroups, providing a more detailed understanding of the condition.
Seven vaginal bacteria were highly accurate for BV diagnosis both in swabs and FVU. Finally a good agreement between Nugent and Claeys was found.

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