Master Thesis

Development of quantitative PCR methods for diagnosis of bacterial vaginosis and vaginal yeast infection

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LiU-IKE-EX—11/02
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Vaginitis is a vaginal infection which affects many women all over the world. The disorder is characterized by an infection of the vaginal area which can cause problems like abnormal vaginal discharge, itching and redness. The two most common causes of vaginitis are bacterial vaginosis and *Candida* vaginitis. The prevalence of bacterial vaginosis in Sweden is around 10-20 % and approximately 75 % of all women will once in their lifetime suffer from vaginal yeast infection.

The clinical symptoms of vaginal infections are not specific and the diagnosis methods of bacterial vaginosis and *Candida* vaginitis are subjective and depended on the acuity of the clinician. Due to the lack of standardized and objective diagnostic tools, misdiagnosis and consequently incorrect treatment may occur. As vaginal infections and symptoms impact greatly of women’s quality of life and vaginitis have been associated with serious public health consequences, it is essential to diagnose and treat the conditions correctly. Hence, there is a great need of better methods of diagnosing these conditions.

The aim of this master thesis was to develop quantitative species-specific real-time PCR assays to use in diagnosing the two most common causes of vaginitis i.e. bacterial vaginosis and *Candida* vaginitis. Potential markers for bacterial vaginosis (*Atopobium vaginae, BVAB2, Gardnerella vaginalis, Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus jenseni, Lactobacillus iners, Megasphaera type 1, Megasphaera type 2, Mobiluncus curtisi, Mobiluncus mulieris and Leptotrichia/Sneathia species*) and *Candida* vaginitis (*Candida albicans, Candida glabrata, Candida parapsilosis and Candida tropicalis*) were chosen. Primers and probes were designed and tested on reference strains and vaginal samples. Single- and multiplex PCR reactions were successfully optimized with the designed oligonucleotides. Furthermore, standard curves with excellent linearity were created and covered more than five orders of magnitude. These developed quantitative species-specific real-time PCR assays will, in a prospective medical validation, quantify 300 vaginal samples from women visiting the RFSU Clinic in Stockholm.

Keywords
Vaginitis, bacterial vaginosis, *Candida, Lactobacillus*, real-time PCR
Preface
This master thesis for a Master of Science degree in Engineering, Chemical Biology was performed at Dynamic Code AB in Linköping, Sweden, between November 2010 and Mars 2011.

Dynamic Code AB, established 2000, is a specialist gene technology company which provides DNA analysis for both private and professional clients. The company is accredited by SWEDAC and all the DNA tests are processed in an ISO17025 accredited DNA testing laboratory.

Dynamic Code AB currently provides comprehensive DNA testing services in several areas: familial relationships, forensics, and lineage analysis in animals, STD, environmental mould and fungus.

In order to protect Dynamic Code AB´s intellectual property rights, primer and probe sequences were not included in this master thesis.

Linköping, Mars 2011

Kristina Eiderbrant
Abstract
Vaginitis is a vaginal infection which affects many women all over the world. The disorder is characterized by an infection of the vaginal area which can cause problems like abnormal vaginal discharge, itching and redness.

The two most common causes of vaginitis are bacterial vaginosis and *Candida* vaginitis. The prevalence of bacterial vaginosis in Sweden is around 10-20 % and approximately 75 % of all women will once in their lifetime suffer from vaginal yeast infection.

The clinical symptoms of vaginal infections are not specific and the diagnosis methods of bacterial vaginosis and *Candida* vaginitis are subjective and depended on the acuity of the clinician. Due to the lack of standardized and objective diagnostic tools, misdiagnosis and consequently incorrect treatment may occur.

As vaginal infections and symptoms impact greatly of women’s quality of life and vaginitis have been associated with serious public health consequences, it is essential to diagnose and treat the conditions correctly. Hence, there is a great need of better methods of diagnosing these conditions.

The aim of this master thesis was to develop quantitative species-specific real-time PCR assays to use in diagnosing the two most common causes of vaginitis i.e. bacterial vaginosis and *Candida* vaginitis.

Potential markers for bacterial vaginosis (*Atopobium vaginae*, BVAB2, *Gardnerella vaginalis*, *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactobacillus iners*, *Megasphaera* type 1, *Megasphaera* type 2, *Mobiluncus curtisi*, *Mobiluncus mulieris* and *Leptotrichia/Sneathia* species) and *Candida* vaginitis (*Candida albicans*, *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*) were chosen. Primers and probes were designed and tested on reference strains and vaginal samples. Single- and multiplex PCR reactions were successfully optimized with the designed oligonucleotides. Furthermore, standard curves with excellent linearity were created and covered more than five orders of magnitude. These developed quantitative species-specific real-time PCR assays will, in a prospective medical validation, quantify 300 vaginal samples from women visiting the RFSU Clinic in Stockholm.
**Sammanfattning**

Infektion i underlivet, vaginit, är något som många kvinnor världen över drabbas av. Sjukdomen kännetecknas av besvärande symtom som onormala vaginale flytningar, klåda och rodnad.

Bakteriell vaginos och svamp är de två vanligaste orsakerna till vaginit. I Sverige drabbas mellan 10-20% av kvinnorna av bakteriell vaginos och ungefär 75% av alla kvinnor får någon gång i livet en svampinfektion i underlivet.

De kliniska symtomen för vaginala infektioner är inte specifika och diagnosmetoderna är subjektiva och beroende av sjukvårdspersonalens kunskaper. Rutinmässiga diagnosmetoder saknas vilket kan leda till felaktiga diagnoser och behandlingar.

Det är viktigt att få rätt diagnos och behandling eftersom vaginala infektioner och symtom har en stark inverkan på kvinnors livskvalitet men även för att vaginit har kunnat kopplas till flera allvarliga hälsorisker. Därför behövs bättre metoder för att diagnostisera bakteriell vaginos och underlivssvamp.

Målet med detta examensarbete var att utveckla kvantitativa, artsuperska realtids-PCR analyser för diagnostisering av de två vanligaste orsakerna till vaginit det vill säga bakteriell vaginos och underlivssvamp.

Potentiella markörer för bakteriell vaginos (Atopobium vaginae, BVAB2, Gardnerella vaginalis, Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus jensenii, Lactobacillus iners, Megasphaera type 1, Megasphaera type 2, Mobiluncus curtisi, Mobiluncus mulieris och Leptotrichia/Sneathia species) och svamp (Candida albicans, Candida glabrata, Candida parapsilosis och Candida tropicalis) valdes ut. Primrar och prober designades mot de utvalda organismerna och testades mot referensstammar och vaginala prover. Primer- och probekoncentrationer optimerades för att kunna analyseras singel- eller multiplex. Standardkurvor med ett brett linjärt spann skapades. Dessa utvecklade kvantitativa, artsuperska realtids-PCR analyser kommer att användas i en framtida medicinsk utvärdering av 300 insamlade vaginala prover från kvinnor som besöker RFSU kliniken i Stockholm.
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1 INTRODUCTION

The state of a normal healthy vagina is largely a function of the bacterial community which is an important first line of defence for the body. *Lactobacillus* species, mainly *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii* are dominant constituents in most women’s vagina worldwide [Ravel *et al.* 2010, Tärnberg *et al.* 2002, Vásques *et al.* 2002, Yamamoto *et al.* 2009, Zhou *et al.* 2010] and defends the vagina by e.g. producing several compounds with antimicrobial activity and thereby creating an inhospitable environment against pathogens.

Despite several vaginal defence mechanisms the vaginal microbiota is sometimes disturbed and there is a change in the normal balance causing symptoms like abnormal or increased vaginal discharge, redness and itching [Granato 2010]. Irritation of vagina caused by inflammation or infection is called vaginitis. Vaginitis is a very common disease for women of reproductive age all over the world but children and postmenopausal women could also be affected.

The two most common causes of vaginitis are bacterial vaginosis and *Candida* vaginitis. The prevalence of bacterial vaginosis in Sweden is around 10-20 % [Moi 1990, Sjukvårdsrådgivningen, 1177 a] and approximately 75 % of all women will once in their lifetime suffer from vaginal yeast infection [Sjukvårdsrådgivningen, 1177 b, Sobel *et al.* 1998].

Bacterial vaginosis is caused by a decrease of lactobacilli concurrent with overgrowth of several fastidious bacterial species which normally could be present in low concentrations in the vagina [Ling *et al.* 2010, Vitali *et al.* 2007]. *Candida* vaginitis is a vaginal yeast infection where *Candida albicans* is commonly the cause of the disorder [Sobel *et al.* 1998]. Bacterial vaginosis and *Candida* vaginitis are extremely common infections in women whose epidemiology and pathogenesis are not clarified.

The clinical symptoms of vaginal infections are not specific and the diagnostic methods of bacterial vaginosis and *Candida* vaginitis are subjective and depended on the acuity of the clinician. Due to the lack of standardized and objective diagnostic tools, misdiagnosis and consequently incorrect treatment may occur. Women should not assume that a new infection is the same as previous and use e.g. over the counter treatment, but rather reconsider the diagnosis and do a thorough examination.

As vaginal infections and symptoms impact greatly of women’s quality of life and vaginitis have been associated with serious public health consequences, it is essential to diagnose and treat the conditions correctly. Hence, there is a great need of better methods of diagnosing these conditions.
1.1 AIM
The aim of this master thesis is to develop bacteria and yeast specific qPCR assays for diagnosing the two most common causes of vaginitis, bacterial vaginosis and *Candida* vaginitis. The literature regarding these two conditions will be studied to find out potentially bacteria and yeast species which can represent these conditions. Species-specific primers and probes will be designed and tested on reference strain and vaginal samples. Optimize the designed oligonucleotides for both single- and multiplex PCR reactions furthermore create standard curves for the prospective medical validation of bacterial vaginosis and *Candida* vaginitis.
2 THEORETICAL BACKGROUND

For eons, microbes and hosts have coevolved which have created mutual benefits for both [Dethlefsen et al. 2007]. Directly after birth microbes are acquired from the environment, creating a complex and usually stable microbiota in and on the body. The symbiosis between humans and their microbiota is of importance for physical as well as psychic health. Functions such as food processing or protection from pathogen infections are dependent of this symbiosis.

2.1 NORMAL VAGINAL FLORA

The microbiota in the human vagina is an important first line of defence since it protects from pathogens and thereby maintains women’s health. Onset of girls’ puberty changes the ecology of the vagina [Granato 2010]. The vaginal epithelium thickens and these epithelia cells produce glycogen which is an important food source for microorganisms. The glycogen rich environment selects for glucose fermenting organisms, mainly lactobacilli. When the glycogen is metabolized the pH in the vagina is changed from nearly physiologic to acidic.

The state of a normal healthy vagina is largely a function of the bacterial community which is an important first line of defence for the body. Lactobacillus species, mainly L. crispatus, L. gasseri, L. iners and L. jensenii are dominant constituents in most women’s vagina worldwide [Ravel et al. 2010, Tärnberg et al. 2002, Vásques et al. 2002, Yamamoto et al. 2009, Yan et al. 2009, Zhou et al. 2010] but other microorganisms are also present [Hyman et al. 2005, Zhou et al. 2007]. Furthermore, the bacterial microbiota is not homogenous throughout the vagina [Kim et al. 2009].

Lactobacillus species controls and defends the vaginal flora from pathogens through several mechanisms. They have a specific adherence to the vaginal epithelium and they also auto-aggregate which create a barrier and consequently obstruct colonization of pathogens [Boris et al. 1998]. Lactobacillus species produce several products which inhibit the growth of pathogens. The vaginal epithelial cells secrete glycogen which lactobacilli ferments to organic acids, mainly lactic acid, creating an acidic environment (generally a pH below 4.5), consequently. This is inhospitable and largely restricts which microorganisms could colonize the vagina [Boskey et al. 2001]. Another product with antimicrobial activity is hydrogen peroxide, a reactive oxygen species that cause oxidative stress and cell damage by reacting with e.g. proteins and nucleic acid [Imlay 2008]. Lactobacilli also produce proteins with bactericidal effect [Jack et al. 1995] which inhibit a wide range of microorganisms but also strains of the same or closely related species.

2.2 VAGINITIS

The stability of the vaginal microbial ecosystem preclude many other organisms but sometimes the vaginal microbiota is disturbed and there is a change in the normal balance causing symptoms like abnormal or increased vaginal discharge, redness and itching [Granato 2010]. Irritation of vagina caused by inflammation or infection is called vaginitis, or vulvovaginitis if both vagina and vulva are inflamed.

The cause of the abnormal vaginal discharge can be divided into three major groups, infectious, non-infectious and chronic vaginitis (Figure 1) [Granato 2010]. Vaginitis is a very common disease for women of reproductive age all over the world. Children and postmenopausal women can also be affected, but not as commonly.

Figure 1 The cause of vaginitis can be divided into three major groups, infectious, non-infectious and chronic vaginitis. Some examples are also given of the three groups.

The largest group is infectious vaginitis involving the majority of the women with this disorder. Bacterial vaginosis and Candida vaginitis are the two most common causes of vaginitis. Bacterial vaginosis is caused by a decrease of lactobacilli concurrent with overgrowth of several fastidious bacterial species which can also be present in small amounts in the healthy vagina [Ling et al. 2010, Vitali et al. 2007]. Candida vaginitis is a vaginal yeast infection where Candida albicans is commonly the cause of this disorder [Sobel et al. 1998]. Another cause belonging to infectious vaginitis is Trichomoniasis which is caused by the protozoan Trichomonas vaginalis. This sexually transmitted vaginitis is the third leading cause of vaginitis worldwide but rare in Sweden [Sjukvårdsrådgivningen, 1177 c].

Non-infectious vaginitis could be due to failure of removing a foreign body like a tampon, presence of genital tumours or use of chemical agents [Granato 2010]. Chronic vaginitis could be due to overgrowth of lactobacilli (cytologic vaginitis) which cause a vaginal pH well below 4.0 or there could be an overgrowth of gram-positive cocci (desquamative inflammatory vaginitis).
2.2.1 Bacterial vaginosis

Bacterial vaginosis is the most common cause of vaginitis worldwide, approximately 50 % of the cases [Allsworth et al. 2007, Joesoef et al. 2001, Morris et al. 2001] but the prevalence varies according to the population studied. In Sweden it accounts for 10-20 % [Moi 1990, Sjukvårdsrådgivningen, 1177 a]. The suffix “itis” in vaginitis implies an inflammation which is not the case why the suffix “osis” is preferred. Bacterial vaginosis is a polymicrobial syndrome with acquisition of several fastidious bacteria (Figure 2) [Fredricks et al. 2005, Ling et al. 2010, Vitali et al. 2007] concurrent with a decrease of lactobacilli, the dominant constituents in normal vaginal flora [Biagi et al. 2009, Ling et al. 2010, Menard et al. 2008, Sha et al. 2005, Tamrakar et al. 2007, Vitali et al. 2007].

Figure 2 Gram-stained vaginal smear from normal micro flora (left) and bacterial vaginosis flora (right). To the left there is a gram stained smear from normal vaginal secretion. The arrow point at Lactobacillus species attached at an epithelial cell. To the right there is a gram stained smear from bacterial vaginosis vaginal secretion. Lactobacillus species are absent and there is an overgrowth of several bacterial species which also coat the epithelial cell (a clue-cell) [Fun with Microbiology, 2010]

The main symptom of bacterial vaginosis is an increased vaginal discharge, which is homogenous, thin, grey and malodorous [Granato 2010]. Fifty percent of the women with bacterial vaginosis, however, are asymptomatic [Klebanoff et al. 2004]. Since many women are affected with bacterial vaginosis and lack symptoms there is a question if it should be considered as a normal variant of the vaginal flora, but bacterial vaginosis has been strongly associated with serious public health consequences such as pelvic inflammatory disease i.e. infection/inflammation of the genital tract [Haggerty et al. 2004, Hillier et al. 1996, Marrazzo et al. 2006 and Ness et al. 2005], preterm labour and birth [Donders et al. 2009, Hillier et al. 1995, Leitch and Kiss 2007, Nelson et al. 2009, Schoeman et al. 2005, Subtil et al. 2002, Svare et al. 2006, Usui et al. 2002, Ugwumadu et al. 2003], late miscarriage [Ugwumadu et al. 2003], postoperative gynaecological complications [Persson et al. 1996, Larsson and Carlsson 2002] and increase risk of acquiring various sexually transmitted disease pathogens such as Neisseria gonorrhoeae [Martin et al. 1999, Wiesenfeld et al. 2003], Trichomonas vaginalis [Martin et al. 1999], Chlamydia trachomatis [Wiesenfeld et al. 2003], HPV (Human papillomavirus) [Watts et al. 2005], HSV-2 (genital herpes simplex virus) [Cherpes et al. 2003] and HIV-1 (Human immunodeficiency virus) [Martin et al. 1999].
Several studies have tried to find epidemiologic factors but the results are many and some are inconsistent, hence there is a transmission disease enigma. Bacterial vaginosis is associated with having new sex partner, vaginal douching and lack of H$_2$O$_2$-producing lactobacilli [Hawes et al. 1996]. The prevalence of the disease is higher in the lesbian group compared with heterosexual couples [Evans et al. 2007]. Bacterial vaginosis has been suggested to be a sexually transmitted disease, since high risk behaviour, such as many sex partners, has been associated with bacterial vaginosis [Bradshaw et al. 2005, Fethers et al. 2008] and women with no sexual experience never suffer from bacterial vaginosis [Fethers et al. 2009]. Other studies, however, have shown bacterial vaginosis in non-sexually experience women [Yen et al. 2003]. Furthermore, treatment of sexual partners has failed when women have got recurrence of bacterial vaginosis [Potter 1999].

There are two possible explanations for the pathogenesis of bacterial vaginosis but its etiology is still unknown (Figure 3). It is not known whether the acquisition of bacteria, associated with bacterial vaginosis or the decrease and depletion of lactobacilli is the primary event initiating bacterial vaginosis [Srinivasan and Fredricks 2008].

![Figure 3 Two possible explanations for the pathogenesis of bacterial vaginosis.](image)

Since there is no obvious symptom of bacterial vaginosis there is several scoring system [Spiegel et al. 1983, Amsel et al. 1983, Nugent et al. 1991, Ison and Hay 2002] to facilitate diagnosing. The two most common are Amsel clinical criteria which are often used in clinic and Nugent scoring which is often employed in research.

Amsel clinical criteria are four criteria where three must be present for making the diagnosis of bacterial vaginosis. The criteria are as follows: vaginal fluid is homogenous, thin and milky; vaginal pH is greater than 4.5 since lactobacilli decrease and do not produce the same amount of products that lower the pH in the vagina; a positive amine test, “whiff test”, dilution of the vaginal secretion with 10% KOH produce a fishy odour; and there should be more than 20% clue cells (Figure 2) i.e. bacterial-coated epithelial cells present when vaginal secretion is examined microscopically.
In the RFSU Clinic (Stockholm) only three of Amsel’s clinical criteria are looked for. The woman is examined and vaginal secretion is obtained for testing. The secretion should be homogenous, grey and thin, the pH should be higher than normal and there should be a positive amine test.

Nugent score is often used in research and is based on analysis of Gram stained vaginal smear. The presence and the relative amount of three bacterial morphotypes are scored. Assessed bacterial morphotypes are large Gram-positive rods (Lactobacillus species), small Gram-negative and Gram-variable rods (Gardnerella vaginalis and Bacteroides species) and curved rods (Mobiluncus species). The Nugent score can range from 0 to 10 where 0 to 3 are normal vaginal flora and a score of 7 to 10 is consistent with BV. A score of 4 to 6 is designated as intermediate vaginal flora which is abnormal but not consistent with bacterial vaginosis.

Bacterial vaginosis is treated orally or topically with antibiotics containing nitromidazole or clindamycin targeting anaerobic organisms. Unfortunately there is high recurrence (more than half of the treated) [Boris et al. 1997, Bradshaw et al. 2006 a, Myer et al. 2006, Larsson et al. 2005] which require repeated and maybe longer treatment and therefore an increased risk of developing Candida vaginitis [Pirotta and Garland, 2006]. Probiotics are under intensive research where isolated lactobacilli is administrated to the vagina to prevent, treat and improve the cure of bacterial vaginosis but with inconsistent result [Ehrström et al. 2010, Hummelen et al. 2010a, Larsson et al. 2008, Senok et al. 2009, Ya et al. 2010]. Further research is thus needed.

2.2.2 Microbiology bacterial vaginosis

Since the new cultivation-independent methods were developed many new organisms have been added to the list of possible infection-agents of bacterial vaginosis. The use of broad range primers has showed a greater bacterial diversity in women with bacterial vaginosis compared to women with normal vaginal flora. The methods and the amount of samples in the studies are varied but in summary bacterial vaginosis is polymicrobial and a very complex infection, consequently.

2.2.2.1 Gardnerella vaginalis

Gardnerella vaginalis is a facultative anaerobic bacterium. This bacterium has been associated with bacterial vaginosis for a long time since it is culturable and one of the three organisms quantified as part of the Nugent score. Gardnerella vaginalis is commonly detected in normal vaginal flora [Bradshaw et al. 2006b, Fredricks et al. 2007, Ling et al. 2010, Vitali et al. 2007, Zozaya-Hinchliffe et al. 2010] therefore its presence is not specific for a pathological condition. On a quantitative basis, however, it is found in significantly higher concentrations in women with bacterial vaginosis [Biagi et al. 2009, Bradshaw et al. 2006b, De Backer et al. 2007, Fredricks et al. 2009, Ling et al. 2010, Menard et al. 2008, 2010, Zariiffard et al. 2002, Vitali et al. 2007, Zozaya-Hinchliffe et al. 2010].
2.2.2.2 Atopobium vaginae


2.2.2.3 Leptotrichia/Sneathia species

The two fastidious bacterial species Leptotrichia amnionii and Sneathia sanguinegens are closely related and can be detected with a single assay. They are strictly anaerobic and belong to the lactic acid producing group of bacteria. These two species seem to be involved in the pathogenesis since their detection is significantly associated with bacterial vaginosis [Fredricks et al. 2005, 2007, Ling et al. 2010, Tamrakar et al. 2007, Zozaya-Hinchliffe et al. 2010]. Several studies [Fredricks et al. 2009, Ling et al. 2010, Zozaya-Hinchliffe et al. 2010] have also found a significant higher concentration of these species in women with bacterial vaginosis compared with women with normal vaginal flora.

2.2.2.4 Megasphaera species

Megasphaera type 1 and type 2 are two Megasphaera-like bacteria which are strictly anaerobic and lactic acid producing bacteria. These species seems to have a role in bacterial vaginosis since they have been found to be associated with bacterial vaginosis [Fredricks et al. 2005, 2007, Ling et al. 2010, Tamrakar et al. 2007]. In several studies [Fredricks et al. 2009, Ling et al. 2010, Vitali et al. 2007, Zozaya-Hinchliffe et al. 2010] these species have been found in significant higher concentrations in women with bacterial vaginosis so they appear to have an infectious involvement in the disease.

2.2.2.5 BVAB2

BVAB2 (bacterial vaginosis-associated bacterium 2) is a relatively newly discovered bacteria. This bacterium is a highly specific indicator of bacterial vaginosis [Fredricks et al. 2005, 2007, Tamrakar et al. 2007] and BVAB2 is found in significant higher concentrations [Fredricks et al. 2009, Zozaya-Hinchliffe et al. 2010] in women with bacterial vaginosis compared with women with normal vaginal flora.

2.2.2.6 Mobiluncus species

Mobiluncus species have like Gardnerella vaginalis been associated with bacterial vaginosis for a long time. They are anaerobic culturable bacteria and one of the three organisms quantified as part of the Nugent score. This was the main reason why they were chosen but Mobiluncus curtisii [Menard et al. 2008] and Mobiluncus mulieris [Zozaya-Hinchliffe et al. 2010] have however been found in significantly higher concentrations in women with bacterial vaginosis compared with women with normal vaginal flora.
2.2.2.7 Lactobacillus species

*Lactobacillus* species is one of the three organisms quantified as part of the Nugent score. These organisms are the major constituents in the vagina of healthy women and believed to promote a healthy ecosystem. A healthy vagina is dominated of one or two *Lactobacillus* species and the four most common are *Lactobacillus crispatus*, *Lactobacillus jensenii*, *Lactobacillus gasseri* and *Lactobacillus iners* [Ravel et al. 2010, Tärnberg et al. 2002, Vásques et al. 2002, Yamamoto et al. 2009, Yan et al. 2009, Zhou et al. 2010].


2.2.3 Candida vaginitis

The second largest group worldwide and probably the most familiar is *Candida* vaginitis also often called vulvovaginal candidiasis/candidosis which is a vaginal yeast infection where the genus *Candida* represents most of the yeast species [Sobel et al. 1998]. Approximately 75 % of all women will once in their lifetime suffer from vaginal yeast infection [Sjukvårdsrådgivningen, 1177 b, Sobel et al. 1998] and nearly half of them will experience a recurrence [Hurley and De Louvois, 1979]. About 5 % of women affected by *Candida* vaginitis will suffer from recurrent infections [Sobel 1985].

*Candida* species overgrow the vagina and sometimes also the vulva but they do not increase on the expense of lactobacilli as in bacterial vaginosis. Women with *Candida* vaginitis also have a vaginal flora dominated by lactobacilli [Biagi et al. 2009, Vitali et al. 2007, Zhou et al. 2009]. In fact, lactobacilli may increase the risk of *Candida* infection [McClelland et al. 2009] contrary to the common hypothesis that lactobacilli prevent colonization of undesirable organisms.

Common symptoms for *Candida* vaginitis are inflammation and irritation such as redness, burning, swelling and pruritus on vulva [Eckert et al. 1998]. The vaginal discharge could be minimal or absent but others report a profuse whitish and curd-like discharge with no odour.

*Candida* vaginitis is a monomicrobial syndrome but its etiology is, like bacterial vaginosis, not known. *Candida* species normally live in small amounts on the human body, e.g. mouth, digestive tract, vagina and skin, in a certain balance with other microorganisms. Many studies have been done to find the cause/causes which trigger overgrowth and development of *Candida* vaginitis but the question is still not clarified. However, some predisposing risk factors have been found. Pregnancy has a significant role in vaginal colonization of *Candida* species and *Candida* vaginitis [Babić and Hukić, 2010]. Nearly 40 % of healthy pregnant women have *Candida* species present in the vagina [Hay and Czeizel, 2007] and there is a risk of vertical transmission [Bliss et al. 2008]. Treatment of *Candida* vaginitis has shown a significant reduction of preterm birth [Czeizel et al. 2004]. Women with diabetes, especially
those with uncontrolled diabetes, have higher prevalence of yeast infection [Amouri et al. 2010, Grigorou et al. 2006]. Overgrowth of Candida species can occur when the body’s immune system is suppressed as in HIV-infected women [Duerr et al. 2003]. Candida vaginitis can develop during or after use of antibiotics [Pirotta and Garland, 2006]. Also the use of contraceptives is a risk factor [Geiger et al. 1995, Grigoriou et al. 2006]. Some reports indicate that genetic factors predispose the susceptibility to colonization of Candida species and Candida vaginitis [Babula et al. 2005, Liu et al. 2005]. Candida infection is significantly associated with great number of female sexual partners in the lesbian group and bisexual women [Bailey et al. 2008].

Candida species seems to be an opportunistic pathogen in the vagina since about 20 % of women are asymptomatic carriers [Barousse et al. 2004] and 70 % of healthy asymptomatic women have low concentrations of Candida species in the genital tract at some point in their lives [Beigi et al. 2004]. Candida species are polymorphic (Figure 4) which mean that they can switch from budding yeast form to filamentous form (hyphal cells) and this ability is thought to be an important virulence factor [Yang 2003].

Figure 4 Yeast species in a gram stained (left) and in a 10 % KOH wet mount (right) vaginal smear. The left picture shows yeast species in a gram stained vaginal smear [Fun with Microbiology, 2010]. The right picture shows yeast species in 10 % KOH wet mount [The Practitioner’s Handbook for the Management of Sexually Transmitted Diseases]. Candida species are polymorphic i.e. both yeast and hyphal cells are produced.

Candida vaginitis is often classified as uncomplicated or complicated [Sobel et al. 1998]. Uncomplicated Candida vaginitis is sporadic and often it is Candida albicans which responds well to antifungal therapy. The woman with uncomplicated Candida vaginitis is often a normal and nonpregnant woman. Complicated Candida vaginitis is recurrent and severe. Recurrent Candida vaginitis refers to four or more episodes in a 12 month period [Sobel et al. 1998]. The microorganisms causing the symptoms may be non-albicans species of Candida and could be nonresponsive to conventional antifungal therapies and/or the woman belongs to a risk group, e.g. immune suppressed women.

Even though culture is considered as the “gold standard” in diagnosing Candida vaginitis [Nyirjesy et al. 1995, Sobel et al. 1998] it is rarely done in clinics but most often in research. In clinics the diagnosis is often based on white vaginal discharge, vulvovaginal pruritus,
normal vaginal pH and a microscopic examination of the vaginal discharge where the vaginal secretion is diluted with saline or 10 % KOH (Figure 4) to facilitate the detection of Candida species.

In the RFSU Clinic (Stockholm) the diagnosis of Candida vaginitis is based on medical history, vaginal examination together with a Candida characteristic vaginal discharge.

Candida vaginitis is treated either orally or topically with antifungal agents such as azoles and polyenes which destroys or prevents the growth of yeast species in nearly 80 % of the uncomplicated cases [Sobel et al. 1998]. Women with recurrent Candida vaginitis need repeated and longer treatment. As in bacterial vaginosis treatment with probiotics are under research and also here with inconsistent results [Falagas et al. 2006, Jeavons 2003, Martinez et al. 2009, Pirotta et al. 2004].

2.2.4 Microbiology Candida vaginitis
The amount of samples in the studies are varied and the conventional systems as cultivation and microscopic examination of vaginal smears are the most common methods to detect Candida species but some studies have used PCR. Candida vaginitis is a monomicrobial disease where some Candida species are more common than others.

2.2.4.1 Candida species


2.2.4.2 Lactobacillus species
Lactobacillus species are the major constituents in normal vaginal flora and women with Candida vaginitis also have a vaginal flora dominated of lactobacilli [Vitali et al. 2007, Zhou et al. 2009, Biagi et al. 2009]. Hence, they are not a potentially marker for Candida vaginitis.
2.3 POLYMERASE CHAIN REACTION TECHNOLOGY

In 1993 Kary Mullis was awarded the Nobel Prize in Chemistry for invented the Polymerase Chain Reaction (PCR) [Madigan et al. 2003]. The principle of PCR technology is a cyclic process (Figure 5) i.e. PCR is performed by a series of temperature cycles, which generates large number of identical copies to a workable amount of a wanted nucleic acid sequence.

![Diagram of PCR cycle]

**Figure 5** Each temperature cycle in PCR involves denaturation, annealing and extension.

The vast advantage of PCR is that this technology can increase the amount of all starting material irrespective of whether they can be cultured or not [Madigan et al. 2003]. This facilitates detection and identification of microbial populations in their natural environment.

2.3.1 Primer

DNA polymerases are enzymes which catalyze addition of nucleotides in the replication [Madigan et al. 2003]. These DNA polymerases cannot begin a new DNA strand therefore a primer with a pre-existing 3′-OH group must be present, at which the DNA polymerase can attach the first nucleotide.

A primer is a short single-stranded DNA sequence which can hybridize to another single stranded DNA sequence [D’haene et al. 2010, Applied Biosystems, 2004]. When amplifying a certain DNA sequence, two primers are needed, one forward and one reverse. The two primers hybridized adjacent to the region of interest and act as starting points for DNA replication by guiding DNA polymerase where to initiate synthesis of the complementary strand.

The optimal length of the primers is approximately 20 bases which are long enough to be specific and short enough to bind easily to the DNA template [D’haene et al. 2010, Applied Biosystems, 2004]. A primer set should be designed to have the same or similar melting temperatures. The GC content should be 30-80 % to ensure stable binding with template. More than 2 G’s or C’s in the 5 last bases at the 3′end should be avoided since it increases the risk of mispriming with G or C rich sequences. Similarly, more than 4 repeats of the same base also increase the risk of mispriming. The secondary structure which could be
formed by intra- or intermolecular interaction affects the template binding. Hairpins, self- and cross dimers reduce the availability of primers for the reaction and hence reduce the amplification of the sequence of interest. Nowadays, there are many commercial programs which consider all these different factors and aid with the primer design.

2.3.2 Real-Time Polymerase Chain Reaction

Real-time PCR was developed 1992 and it is a fluorescence detecting thermal cycler, measuring the PCR amplification as it occurs [Strachan and Read 2004]. The thermal cycler monitors the fluorescence of probes or dyes each cycle and the fluorescence signal is proportional to the amount of amplicons. When assuming that there is a doubling of amplicons each cycle, it is possible to calculate the initial number of the wanted sequence, so theoretically:

\[ N = N_0 \times 2^n \]

\[ N = \text{number of amplified products}, \quad N_0 = \text{initial number of products}, \]

\[ n = \text{number of amplification cycles}. \]

To identify the amount of DNA various detection chemistries are available with non-specific fluorescent dyes or sequence-specific fluorescent labelled probes. Non-specific fluorescent dyes e.g. SYBR® green bind to any double stranded DNA and fluoresce strongly when bound to DNA [Applied Biosystems, 2005]. Probes e.g. TaqMan® are labelled oligonucleotides which hybridize to a specific single stranded DNA sequence. These probes depend on Förster Resonance Energy Transfer (FRET). Using probes increases the specificity of the assay.

The advantage with sequence-specific fluorescent labelled probes is that they can be used in multiplex PCR assays i.e. simultaneous amplifying multiple sequences in a single tube and detected parallel, if each specific probe has a unique reporter dye.
2.3.3 TaqMan Chemistry

One of the chemistries which are available for real-time PCR is TaqMan® [Applied Biosystems, 2005]. A TaqMan probe has a fluorescent reporter dye conjugated at the 5´end and a quencher dye conjugated at the 3´end. The reporter dye and the quencher dye are so close to each other that no light emit upon irradiation i.e. FRET. These probes hybridize to single-stranded DNA, downstream the primer sites during the annealing step (Figure 6). The primers are then elongated by the Taq DNA polymerase. As the bound probe is reached the polymerase, which also has a 5´- 3´exonuclease activity, hydrolyzes the probe and the reporter dye and the quencher are separated from each other. The reporter dye is no longer quenched i.e. FRET no longer occurs and emission of fluorescence can be detected by the thermal cycler. Each free reporter dye represents a new DNA strand so the fluorescence signal is proportional to the amount of amplicons which permit a quantitative measurement.

![Figure 6 Overview of TaqMan chemistry](image)

The probe with a reporter dye and a quencher dye anneals to the target sequence. FRET occurs due to the proximity of the dyes.

The polymerase with the 5´- 3´ exonuclease activity extends the primer and cleaves the probe. FRET no longer occur and the fluorescent reporter emits light. Each free reporter dye represents a new DNA strand.

Figure 6 Overview of TaqMan chemistry [Bass et al. 2010].
2.3.4 Real-time PCR output

The logarithmic change in the normalized reporter signal (Delta Rn) is plotted versus the cycle number in an amplification plot, to easily identify the log-linear range of the curve (Figure 7) [Applied Biosystems, 2008]. The normalized reporter signal is defined as the intensity of the reporter signal divided by the intensity of an internal passive reference signal, e.g. ROX, which allows correction of well-to-well variation. Since the amount of amplicons should double in each cycle the reporter signal should increase exponentially but in reality one or more of the reaction components become limited and the amplification eventually reaches a plateau. The amplification plot consists of an exponential phase and a plateau phase, consequently.

![Diagram of Delta Rn vs Cycle](image)

**Figure 7 Real-time PCR output of Atopobium vaginae.** The real-time PCR was run for 40 cycles. The amplification plot shows the exponential and plateau phases. Ct is the point where the amplification plot crosses the fixed threshold.

Even though amplicons increase exponentially until the plateau phase, it is not seen in the beginning of the amplification plot since there are not enough of PCR products to yield a detectable reportersignal [Applied Biosystems, 2008]. These initial PCR cycles, where there is no detectable increase of fluorescence, are set to be the baseline (not shown).

Somewhere in the linear region of the amplification plot an arbitrary fluorescence level, a fixed threshold, can be chosen. The cycle at which the amplification plot crosses the threshold is called the threshold cycle (Ct) or the crossing point (Cp) [Applied Biosystems, 2008]. The Ct value increases with a decreasing amount of templates and the Ct value is an essential component to use in quantification of samples with unknown concentrations.
2.3.5 Quantitative PCR
Real-time PCR could be used to only detect a specific DNA sequence, called qualitative PCR, but also to detect and measure the amount, called quantitative PCR [Applied Biosystems, 2008].

One common strategy used in quantification is the standard curve method [Applied Biosystems, 2008]. In this method a standard curve is used as a reference standard for extrapolating Ct values from DNA amplification of samples with unknown concentrations. The standard curve is generated from a dilution series, often 10-fold dilution, constructed from a “reference” sample with known concentration (Figure 8).

![Delta Rn vs Cycle](image)

**Figure 8 Amplification plots of Lactobacillus species in a 10-fold dilution series.** 6 different concentrations in the same amplification plot.

The obtained Ct values from the dilution series are plotted versus the logarithm of the template amount which gives a straight line, a standard curve (Figure 9), and extrapolation of Ct values from samples with unknown concentrations allow the samples to be quantified [Applied Biosystems, 2008] but the Ct values must be within in the dynamic range where the standard curves are linear, for accurate quantification.
Figur 9 Standard curve of Lactobacillus species. The 6 Ct values obtained from the dilution series are plotted versus the logarithm of the template amount which generates a standard curve.

The slope in a standard curve is an important parameter which indicates the efficiency of the real-time PCR [Applied Biosystems, 2008]. If the amount of PCR product is doubled each cycle the efficiency is 100 % which corresponds to a slope of -3,32.

$$PCR\ efficiency\ (%) = (10^{-\text{slope}} - 1) \times 100$$

The real-time PCR should preferably have an efficiency between 90 % and 110 %, i.e. a slope between -3,58 and -3,1 to be accurate.

Another important parameter obtained from the standard curve is the correlation coefficient, $R^2$ [Applied Biosystems, 2008]. It is a statistical term that gives information of the goodness-of-fit in the model. It represent how well the experimental values in the standard curve fit the obtained regression line and it should be > 0,99 to be precise.

If some of the parameters are varying or if there is a failure of amplifying the desired product, singleplex or multiplex, there are some optimization strategies to take into consideration e.g. the concentrations of primers, probes, MgCl$_2$ or the cycling temperatures [Applied Biosystems 2005, Markoulatos et al. 2002].
3 METHODOLOGY

3.1 CHOSEN CANDIDATE SPECIES

According to the literature there is no single bacterium or yeast species causing bacterial vaginosis and Candida vaginitis respectively. Organisms which are associated with bacterial vaginosis, Candida vaginitis or vaginal health were chosen and are listed in Table 1.

Table 1 Bacteria and yeast species, GenBank numbers for each microorganism used in alignment furthermore the reference strains used in primer and probe validation.

<table>
<thead>
<tr>
<th>Species</th>
<th>GenBank nr</th>
<th>Reference strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atopobium vaginae</td>
<td>AY738658, EF120360</td>
<td>CCUG 38953T</td>
</tr>
<tr>
<td>BVAB2</td>
<td>AY724740</td>
<td>Ordered oligonucleotide</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>FM178299, AB365317</td>
<td>CCUG 44147</td>
</tr>
<tr>
<td>Candida glabrata</td>
<td>AB032177, AF167993</td>
<td>CCUG 44136</td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>AF455530, AF287909</td>
<td>CCUG 32995T</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>AF287910, AF268095</td>
<td>CCUG 34274T</td>
</tr>
<tr>
<td>Gardnerella vaginalis</td>
<td>M58744, GQ179720</td>
<td>CCUG 3717T</td>
</tr>
<tr>
<td>Lactobacillus crispatus</td>
<td>AF257097</td>
<td>CCUG 30722T</td>
</tr>
<tr>
<td>Lactobacillus gasseri</td>
<td>AF243165</td>
<td>CCUG 31451T</td>
</tr>
<tr>
<td>Lactobacillus jensenii</td>
<td>AF243176</td>
<td>CCUG 35572T</td>
</tr>
<tr>
<td>Lactobacillus iners</td>
<td>AY283275</td>
<td>CCUG 28746T</td>
</tr>
<tr>
<td>Megasphaera, type 1</td>
<td>AY738672, GU402193</td>
<td>CCUG 45952</td>
</tr>
<tr>
<td>Megasphaera, type 2</td>
<td>AY738697</td>
<td></td>
</tr>
<tr>
<td>Mobiluncus curtisii</td>
<td>AJ576088, EF428974</td>
<td>CCUG 17993</td>
</tr>
<tr>
<td>Mobiluncus mulieris</td>
<td>AJ57608, AY738684</td>
<td>CCUG 20071T</td>
</tr>
<tr>
<td>Leptotrichia amnionii</td>
<td>AY724742</td>
<td>CCUG 41628</td>
</tr>
<tr>
<td>Sneathia sanguinegens</td>
<td>AY738659</td>
<td></td>
</tr>
</tbody>
</table>

3.2 DESIGN OF PRIMERS AND PROBES

Primers and probes were designed, on the basis of previously published DNA sequences, to target highly variable i.e. species-specific regions of the 16S rDNA, for the bacterial species and the section between 18S and 28S rDNA, the ITS 1 - 5.8S - ITS 2 region, for the yeast species. Sequences for each species were obtained from GenBank and by multiple alignments with CLC Sequence Viewer 6.4 (CLC bio), target sites were identified. Primers and probes were chosen using Primer Express® Software v3.0 (Applied Biosystems) and to ensure their specificity they were submitted to BLAST, NCBI database (NCBI: http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Lactobacillus species were aligned and a single set of primers and one probe were designed to hybridise to all four. The two Mobiluncus species were also aligned together for design of a single set of primers and one probe. Primer set and a probe for detecting Megasphaera species type 1 and type 2 was also design to detect Megasphaera micronuciformis so the primer set and the probe could be validated on a Megasphaera micronuciformis strain. The four Candida species have the same probe and reverse primer but species-specific forward primers. BVAB2 is a novel and culture-resistant bacterium in the Clostridiales order and
there is no reference strain to validate the probe and primer set on, therefore a custom made oligonucleotide (Scandinavian Gene Synthesis AB) was ordered for this purpose. The other bacterial species have species-specific primers and probes. All primers and probes were custom made by Applied Biosystems where the probes were duel-labelled TaqMan® MGB™ Probes, differently labelled with FAM™, NED™ or VIC®.

3.3 DNA EXTRACTION
All microorganisms, except BVAB2, were purchased from CCUG (Culture Collection, University of Göteborg, Sweden) as indicated in Table 1. Each freeze-dried reference strain was resuspended in 500 µL ddH₂O (double distilled water). 80 µL of the resuspended organism was homogenised (TissueLyser, Qiagen) for 2 minutes at 30 Hz and then DNA was extracted with QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s protocol. Microorganisms replaced with ddH₂O served as negative controls.

The custom made oligonucleotide for validation of BVAB2’s primer set and probe, was diluted to 10 fM with Tris buffer before validation and optimization.

Vaginal samples were extracted with ZR-96 Genomic DNA Kit (Zymo Research) according to the manufacturer’s protocol with the exception of an additional homogenization step (TissueLyser, Qiagen), 2 minutes at 30 Hz, in the beginning of the extraction. Vaginal samples replaced with ddH₂O served as negative controls.

3.4 QUANTITATIVE POLYMERASE CHAIN REACTION
PCR amplification was performed in 96-well flat top PCR microplates or 0,2 ml thin wall PCR tubes (Axygen Scientific) on 7300 Real-Time PCR System (Applied Biosystems) and the two-step PCR condition was as follows: initial denaturation at 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles of amplification; 0,15 minutes at 95°C (denaturation) and 1 minute at 60°C (simultaneous annealing and extension). No-template controls were performed in each PCR run for detection of contamination.

3.5 VALIDATION OF PRIMERS AND PROBES
Each 15 µl PCR reaction contained 1X PerfeCTa™qPCR (Quanta Biosciences), 300 nM each of forward and reverse primer, 200 nM probe and 3 µl sample.

Samples which designed oligonucleotides were tested on:

- each species individually
- a sample containing all the species (each species diluted 1:1000)
- a pooled vaginal sample (50 vaginal samples were pooled to create a sample with a natural matrix and all the chosen species present)
- 3 vaginal samples
3.6 OPTIMIZATION OF PRIMERS AND PROBES
Primer and probe concentrations were investigated without modifying any other parameters. Three different primer concentrations of 75 nM, 150 nM, 300 nM and three different probe concentrations of 50 nM, 100 nM and 200 nM were valued. The pooled vaginal sample served as DNA template. The optimized oligonucleotides’ concentrations in the singleplex assays are listed in Table 2.

3.7 MULTIPLEX
Two triplex-assays were constructed where BVAB2, *Megasphaera* species and *Mobiluncus* species were the targets in one assay and *Gardnerella vaginalis, Atopobium vaginae* and *Leptotrichia/Sneathia* species were the targets in the other.

To determine whether all reactions in the triplex assays proceeded independently of each other, single- and triplex assays for chosen species were run in the same plate. Initially the same optimized primer concentrations from singleplex were used and if needed, adjusted. Also different template concentrations were tested in the multiplex evaluation, where high concentration had a Ct value about 20 and low concentration had a Ct value about 30. The optimized primer concentrations in the multiplex assays are listed in Table 2.

3.8 STANDARD CURVES
After optimization of primer and probe concentrations, standard curves were created by a 10-fold serial dilution in eight steps. *Candida* species and *Lactobacillus* species were run singleplex while the species in each triplex assay were run together.

For each method the Ct values from the dilution series of respective species were plotted against the logarithmic template amount (arbitrary unit) to create each method’s standard curve. The template amounts in the dilution series were not quantified since it is a relative method. All parameters obtained from the standard curves are listed in Table 3.
4 RESULT

4.1 VALIDATION OF PRIMERS AND PROBES
Each species-specific primers and probes were successfully singleplex tested in samples with only the target template and samples with all the chosen species within. Additionally the designed primer and probes were tested for possible cross-reactivity with some of the other chosen species and there were no amplification. The amplification plot of the pooled vaginal sample (Figure 10) showed that all chosen species were represented. This sample was used for further optimization since it comprises not only the chosen markers but also other molecules present in a vaginal sample.

![Amplification plot of detected species in the pooled vaginal sample.](image)

Figure 10 Amplification plots of detected species in the pooled vaginal sample. All chosen species were represented in high concentrations (data not shown for BVAB2) and could be used for further validation.

To elucidate that the designed oligonucleotides were specific and not cross-reacted with something else in vaginal fluid, vaginal swabs from three volunteers (Dynamic Code AB) were tested. *Lactobacillus* species (Figure 11) were present in high concentrations which is synonymous with healthy vaginal flora. Some other species were present in very low numbers except in one sample where relative high concentrations of *Candida* species were found.
Figure 11 Amplification plots of the three vaginal samples. The plot show high concentrations of *Lactobacillus* species and low concentrations of *Mobiluncus* species, *Gardnerella vaginalis* and *Megasphaera* species. One sample had high concentration of *Candida* species but none of the three participants had any symptoms.

4.2 OPTIMIZATION OF PRIMERS AND PROBES (SINGLEPLEX AND MULTIPLEX)

The PCR reactions were first optimized singleplex (Table 2). Concentrations of primers and probes were optimized with lowest level of concentration but still the same Ct value. The designed primers and probes were successfully tested and optimized, hence suitable for the development of multiplex assays.

Table 2 The optimized concentrations of primers and probes in single- and multiplex amplification.

<table>
<thead>
<tr>
<th>Species</th>
<th>Concentration primer (singleplex)</th>
<th>Concentration primer (multiplex)</th>
<th>Concentration probe</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Atopobium vaginae</em></td>
<td>75nM</td>
<td>300nM</td>
<td>100nM</td>
</tr>
<tr>
<td><em>BVAB2</em></td>
<td>75nM</td>
<td>75nM</td>
<td>100nM</td>
</tr>
<tr>
<td><em>Candida species</em></td>
<td>75nM</td>
<td>75nM</td>
<td>50nM</td>
</tr>
<tr>
<td><em>Gardnerella vaginalis</em></td>
<td>75nM</td>
<td>600nM</td>
<td>100nM</td>
</tr>
<tr>
<td><em>Lactobacillus species</em></td>
<td>75nM</td>
<td>75nM</td>
<td>100nM</td>
</tr>
<tr>
<td><em>Megasphaera species</em></td>
<td>75nM</td>
<td>75nM</td>
<td>100nM</td>
</tr>
<tr>
<td><em>Mobiluncus species</em></td>
<td>75nM</td>
<td>75nM</td>
<td>100nM</td>
</tr>
<tr>
<td><em>Leptotrichia/Sneathia</em></td>
<td>150nM</td>
<td>300nM</td>
<td>100nM</td>
</tr>
</tbody>
</table>

*Candida* species and *Lactobacillus* species were chosen not to be multiplex and the choice of which species assembled in each multiplex assay were at random. To ensure that results obtained multiplex were accurate and amplification proceeded independently of each other, singleplex and multiplex assays were run in the same plate to compare the Ct values.
The multiplex assay with *Mobiluncus* species, BVAB2 and *Megasphaera* species worked accurately at first try. The plot nearly overlapped completely (Figure 12) and the oligonucleotide concentrations did not require any adjustment.

![Amplification plots](image)

**Figure 12** Amplification plots of *Mobiluncus* species, BVAB2 and *Megasphaera* species, singleplex and triplex. The plots from single- and multiplex nearly overlapped.

In the other multiplex assay there were problems since the result from single- and multiplex PCR reaction showed that the amount of template strongly influenced the outcome of the reaction. To overcome these problems various combinations of primer concentrations in the reactions were tested. The best result was obtained when primer set detecting *Gardnerella vaginalis* were 8 times higher, *Atopobium vaginalis* were 4 times higher and *Leptotrichia/Sneathia* species were doubled (Table 2) compared to the singleplex concentrations. When these oligonucleotides concentrations were used the Ct values did not differ significantly but the appearance of the amplification plot changed (Figure 13).
Figure 13 Amplification plots of *Gardnerella vaginalis* (left) and *Leptotrichia/Sneathia* species (right), singleplex and triplex. The amplification plots shows when *Gardnerella vaginalis* and *Leptotrichia/Sneathia* species are triplex, the multiplex assay change in appearance compared with singleplex, but not significantly in Ct.

4.3 STANDARD CURVES

Standard curves were successfully created and the obtained values from each method are compiled in Table 3. The quantitative PCR assays were linear and covered more than five orders of magnitude.

Table 3 The values; slope, intercept, $R^2$ and dynamic range from each specie’s standard curve

<table>
<thead>
<tr>
<th>Species</th>
<th>Slope</th>
<th>Intercept</th>
<th>$R^2$</th>
<th>Dynamic range</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Atopobium vaginae</em></td>
<td>-3,126210</td>
<td>35,849857</td>
<td>0,999542</td>
<td>1:10 to 1:10⁵</td>
</tr>
<tr>
<td>BVAB2</td>
<td>-3,388264</td>
<td>28,951017</td>
<td>0,997756</td>
<td>1:10³ to 1:10⁷</td>
</tr>
<tr>
<td><em>Candida species</em></td>
<td>-3,655168</td>
<td>39,678467</td>
<td>0,999889</td>
<td>1:10 to 1:10⁷</td>
</tr>
<tr>
<td><em>Gardnerella vaginalis</em></td>
<td>-3,105546</td>
<td>35,663246</td>
<td>0,997832</td>
<td>1:1 to 1:10⁷</td>
</tr>
<tr>
<td><em>Lactobacillus species</em></td>
<td>-3,584025</td>
<td>42,711475</td>
<td>0,999067</td>
<td>1:1 to 1:10⁵</td>
</tr>
<tr>
<td><em>Megasphaera species</em></td>
<td>-3,44703</td>
<td>40,947548</td>
<td>0,998578</td>
<td>1:1 to 1:10⁵</td>
</tr>
<tr>
<td><em>Mobiluncus species</em></td>
<td>-3,472193</td>
<td>40,821537</td>
<td>0,998365</td>
<td>1:1 to 1:10⁷</td>
</tr>
<tr>
<td><em>Leptotrichia/Sneathia</em></td>
<td>-3,288928</td>
<td>35,231686</td>
<td>0,998639</td>
<td>1:1 to 1:10⁸</td>
</tr>
</tbody>
</table>
5 DISCUSSION AND CONCLUSION

The aim of this master thesis was to develop bacteria and yeast specific qPCR assays for diagnosis of the two most common causes of vaginitis, bacterial vaginosis and Candida vaginitis. The choice of potential markers was based on literature surveys. Species-specific primers and probes were designed to target 13 bacteria and 4 yeast species for diagnose these two conditions. The designed primers and probes were successfully tested and optimized, both single- and multiplex. Furthermore, the created standard curves have excellent linearity and covered more than five orders of magnitude. These developed quantitative species-specific real-time PCR assays will, in a prospective medical validation, quantify 300 vaginal samples from women visiting the RFSU Clinic in Stockholm.

The vaginal microbota has an important task to maintain women’s health. Constitutes dominating the vagina worldwide seem to be Lactobacillus species and their ability to create an inhospitable environment preclude many other organisms. Influences or disruption of this microbial barrier can result in vaginitis. Bacterial vaginosis is the most common cause of vaginal infections and it accounts for 22–50 % [Andersson et al. 2004] of all vaginitis cases. It is a polymicrobial disease where molecular studies have identified many novel species which previously were undetected by cultivation-dependent methods.

Gardnerella vaginalis has been associated with bacterial vaginosis for a long time since it is culturable. This bacterium is often present in healthy vaginas [Bradshaw et al. 2006b, Fredricks et al. 2007, Ling et al. 2010, Vitali et al. 2007, Zozaya-Hinchliffe et al. 2010] like Atopobium vaginae [Menard et al. 2008, Verhelst et al. 2004] which was isolated 1999 in a Swedish woman [Rodriguez Jovita et al. 1999]. Since both are commonly detected with PCR in both healthy women and women with bacterial vaginosis, quantitative methods are needed. In a recent study by Menard et al. (2010) it was shown that the cut-off for these bacteria must be set at a high enough level to avoid false-positive results. Both Amsel criteria and Nugent score were applied on the 163 vaginal samples, from pregnant women with abnormal vaginal discharge, to assess the bacterial vaginosis status. Twenty-five women had bacterial vaginosis according to at least one of the reference methods and all of them had high concentrations of Gardnerella vaginalis (≥10^9/mL) and/or Atopobium vaginae (≥10^8/mL) and the combination of these two species gave a sensitivity of 100 % and a specificity of 93 %. None of the women with normal flora had these high values but the 9 samples which were classified as intermediate by Nugent score and normal with Amsel criteria had.

The diagnosis methods are many, inconsistent and no one are ideal. Both Amsel criteria and Nugent score have subjective components, such as microscopic investigation and olfaction. The advantage of the Amsel criteria is that it classifies all samples as bacterial vaginosis or normal vaginal flora. Nugent score classifies an intermediate vaginal flora which in some studies not included in the result, i.e. just normal and bacterial vaginosis samples are compared. This intermediate flora would be interesting to follow up to elucidate if it is a
variant of normal flora or precursor of bacterial vaginosis. The outcome of this would help when designing molecular tools.

Neither Amsel criteria nor Nugent score identify the pathogens in the vaginal samples. In Nugent score three morphologies counts but the presumed organisms are not ensured with other methods. *Mobiluncus* species are one of the three organisms quantified as part of the Nugent score but since the new cultivation-independent technology emerged these species are rarely detected [Hyman *et al.* 2005, Fredricks *et al.* 2005, 2007, Ling *et al.* 2010, Menard *et al.* 2008, Zozaya-Hinchliffe *et al.* 2010]. *Mobiluncus* species is observed in vaginal smears but the results with PCR seldom mention an association. Maybe this is because it is not *Mobiluncus* species seen in microscopy but rather other bacterial vaginosis-associated bacteria with the same morphology [Fredricks *et al.* 2005]. However, since these species are an important member in Nugent score they were chosen as potential markers.

BVAB2 and *Megasphaera* species are fastidious and strictly anaerobic bacteria which individually are sensitive and specific indicators of bacterial vaginosis but have also together shown promising result in PCR diagnosing bacterial vaginosis [Fredricks *et al.* 2005, 2007, Mitchell *et al.* 2009]. Fredricks *et al.* (2007) found in the study, with 183 samples from normal vaginal flora and 81 samples from bacterial vaginosis affected women (Amsel criteria), that the combination of detecting either BVAB2 or *Megasphaera* type 1 produces high sensitivity and specificity, 99 % and 89 % respectively.

The strictly anaerobic *Leptotrichia/Sneathia* species appears to be an infectious component in the disease since relatively new PCR studies [Fredricks *et al.* 2009, Ling *et al.* 2010, Zozaya-Hinchliffe *et al.* 2010] have found a significant higher concentration of these species in samples demonstrating bacterial vaginosis compared with normal.

All these chosen species seem to be etiologic agents in bacterial vaginosis. Fredricks *et al.* (2009) study the changes in vaginal flora with quantitative PCR, after antibiotic therapy of 24 women with bacterial vaginosis (Amsel criteria). All chosen pathogen markers (except *Mobiluncus* species which were not included in the study) were 1000 to 10 000 fold reduced and nearly eradicated when these women were cured and this difference were highly significant for all (*Gardnerella vaginalis* (P < 0,0001), *Atopobium vaginae* (P < 0,0001), BVAB2 (P = 0,0004), *Megasphaera* species (P < 0,0001) and *Leptotrichia/Sneathia* species (P = 0,0002)). Of the *Lactobacillus* species only *Lactobacillus crispatus* were included in the above study and 10 of the bacterial vaginosis affected women had a vaginal flora which included this species. These 10 women had a significantly increase of this lactobacilli as they were cured.

*Lactobacillus iners* is one major constituent in women’s vaginal flora [Ravel *et al.* 2010, Tärnberg *et al.* 2002, Vásques *et al.* 2002, Yamamoto *et al.* 2009, Yan *et al.* 2009, Zhou *et al.* 2010] which demonstrate how misleading cultivation-dependent methods could be. This was detected as recently as 1999 [Falsen *et al.* 1999] when it was found to grow on specialized
media. This bacterium is a dilemma since studies have shown inconsistent results. *Lactobacillus iners* has been found to be robust lactobacilli and not affected of the bacterial vaginosis status [Fredricks *et al.* 2007, Tamrakar *et al.* 2007, Zozaya-Hinchliffe *et al.* 2010]. However, Ling *et al.* (2010) found a markedly decrease of *Lactobacillus iners* in women with bacterial vaginosis. Quite contrary, other studies have shown a positive correlation between *Lactobacillus iners* and *Gardnerella vaginalis* [De Backer *et al.* 2007, Yan *et al.* 2009]. Hummelen *et al.* (2010b), Jakobsson and Forsum (2007) and Ferris *et al.* (2007) found that this bacterium was the first species to be established after treatment of bacterial vaginosis. Menard *et al.* (2008, 2010) have also found, when one primer set targeted all the four lactobacilli in quantitative PCR, there were a significant lower concentration of lactobacilli in samples demonstrating bacterial vaginosis compared with normal vaginal flora, hence one primer set detecting all four species are applicable.

*Candida* vaginitis accounts for 17–39 % [Andersson *et al.* 2004] of all vaginitis cases. In most studies [Babić and Hukić, 2010, Fan *et al.* 2008, Grigoriou *et al.* 2006, Mahmoudi Rad *et al.* 2011, Moreira and Paula 2006, Pakshir *et al.* 2007, Pirotta *et al.* 2006] conventional systems as culture and microscopic examination of vaginal smears are the preferred method to identify *Candida* species but some studies have used PCR [Mandviwala *et al.* 2010, She *et al.* 2008, Trama *et al.* 2005, 2008, Vermitsky *et al.* 2008, Weissenbacher *et al.* 2009]. The dilemma with *Candida* species is how to interpret the positive result with PCR - is there colonization or a true infection? Unfortunately no studies were found for correlation of quantitative PCR and true infection. Weissenbacher *et al.* (2009) investigated the relationship of 104 symptomatic women with a history of recurrent *Candida* vaginitis and detection of *Candida* species by culture and PCR. PCR was more sensitive in detecting *Candida* species, than cultivation. At the same time this study shows how misleading clinical signs are. Only 31 % (culture) and 42,3 % (PCR) of the samples tested positive for *Candida* species and the symptoms must therefore be caused by something else. Also Jindal *et al.* (2006) demonstrated that *Candida* vaginitis cannot be accurately diagnosed by clinical symptoms. Only 23 % of 400 symptomatic women had positive culture for *Candida* species.

The women’s immune system is constantly facing the challenge of discriminating between pathogenic and symbiotic microorganisms in the vagina. The vaginal flora is dynamic and fluctuation in the microflora is normal. Furthermore, some bacteria and yeast species can be present in low concentrations which make it difficult to define molecular tests. One major challenge in developing a molecular test is to determine the limits, the cut-off values, between healthy and altered vaginal floras. The three vaginal samples from women without symptoms in this thesis (Figure 11) showed the expected high concentrations of lactobacilli and low titers of some other bacteria. Thus, the diagnosis, in relation to bacterial vaginosis would probably be healthy vaginal floras. However, one sample had relative high concentrations of *Candida* species but all participating women consider themselves healthy. This will be a challenging problem to solve with a molecular test since many women are asymptomatic carriers of potential pathogenic species. The response to the presence of
certain microorganisms e.g. *Candida* species or *Gardnerella vaginalis*, in the vagina seems to determine whether or not a woman is symptomatic. This phenomenon raises the interesting question whether treatment should be based on a positive molecular test alone. Perhaps the result from PCR must also be interpreted together with symptoms.

It is of importance to find out what defines a healthy and altered vaginal flora for the development of accurate molecular diagnostic methods. Today there is no exact definition of normal vaginal flora but maybe The Human Microbiom Project [The NIH Common Fund 2011, The Human Microbiome Project 2011] will find the answer when the vagina´s microbial community is characterized and its role in health and disease is analyzed.

The research regarding bacterial vaginosis and *Candida* vaginitis, whose etiology and pathogenesis are not fully known, illustrate how complex and complicated these diseases are. There is a need of new methods of confirming their diagnosis since clinical symptoms are not specific and they demand different treatment regimens.

Real-time quantitative PCR is an established tool for DNA quantification. It permits the amplified sequence to be identified and quantitative measured immediately since no post-PCR processing is required. Multiplex assays simultaneous detect multiple organisms from a sample and diagnosis or exclusion of a disease is possible. Since there are several possible infectious agents and their concentrations correlate to bacterial vaginosis and *Candida* vaginitis, real-time quantitative PCR is an excellent diagnostic tool as the designed assays in this thesis simultaneous detect and quantifies multiple species.

In summary, real-time PCR assays were successfully developed for 17 species which are associated with bacterial vaginosis, *Candida* vaginitis or vaginal health. This method is rapid and provides both detection and quantification of key species for characterization of vaginal samples in the prospective medical validation.
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7 REFERENCES


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