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The vaginal microbiome: New information about genital tract flora using molecular based techniques

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Abstract

Vaginal microbiome studies provide information which may change the way we define vaginal flora. Normal flora appears dominated by one or two species of *Lactobacillus*. Significant numbers of healthy women lack appreciable numbers of vaginal lactobacilli. Bacterial vaginosis (BV) is not a single entity, but different bacterial communities or profiles of greater microbial diversity than is evident from cultivation-dependent studies. BV should be considered a syndrome of variable composition which results in different symptoms, phenotypical outcomes, and responses to different antibiotic regimens. This information may help to elucidate the link between BV and infection-related adverse outcomes of pregnancy.

INTRODUCTION

In normal pregnancy, the resident vaginal microbial flora is thought to provide protection against infection by a number of different mechanisms.[1] In non-pregnant women, the presence of bacterial vaginosis (BV) is associated with an increased risk of upper genital tract and sexually transmitted infections[2-4] and acquisition of HIV.[5-9] In pregnancy, BV increases the risk of post-abortal sepsis,[10] early miscarriage[11] recurrent abortion,[12] late miscarriage,[12;13] preterm prelabor rupture of membranes (PPROM),[14] spontaneous preterm labor (SPTL) and preterm birth (PTB),[13;15-26] histological chorioamnionitis[27;28] and postpartum endometritis.[29;30] As a result, abnormal vaginal flora may predispose to ascending colonization of the genital tract, infiltration of the fetal membranes, microbial invasion of the amniotic cavity[31] and fetal damage.[32;33] Preterm birth of infectious etiology

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is associated with high perinatal mortality and morbidity,[34-36] and a high cost to the healthcare system.[37-39] Much of our knowledge about the composition of the vaginal microbial flora comes from qualitative and semi-quantitative descriptive studies using cultivation-dependant techniques.[40-44] In recent years, the development and introduction of cultivation-independent molecular-based techniques have provided new information about the composition of normal vaginal flora as well as abnormal colonization of the genital tract which complements existing knowledge from cultivation-dependent techniques. This review seeks to inform the busy obstetrician about the background to these new developments and what additional information they provide. These new data might help to elucidate the composition and function of normal flora, and appreciate the microbial diversity, diagnosis, and treatment assessment of abnormal genital tract flora which may affect the outcome of pregnancy.

THE HUMAN MICROBIOME PROJECT

During the conduct of the human genome project, scientists estimated that the number of genes in the human genome that would be needed to code for the proteins required to sustain human physiology, would be approximately 100,000. The researchers were somewhat humbled to find only 20,000 protein coding genes, similar to that of the fruit fly. However, if one considers the human to be a super-organism, (the sum of all human genes plus those of the micro-organisms in, or on us), then the human genome plus the microbial genome [microbiome] which amounts to the collective genome of the symbionts may be considerably more than 100,000 genes. This is supported by the fact that the human microbiome provides traits which the human organism has not needed to evolve for itself.[45;46] The NIH in its roadmap for medical research has committed $100,000,000 to the Human Microbiome Project and plans to investigate five sites – the oral and nasal cavities, the gastrointestinal and genito-urinary tracts and the skin. The genital tract microbiome will be studied in health and disease and whether the function of the microbiome (metagenome) can be manipulated to influence physiology and treat disease. The core microbiome, as well as the variable microbiome (influenced *inter alia* by host lifestyle, genotype, immune response, environment, pathophysiology and transient community members) will be assessed spatially and temporally,[47] and different sub-sites will be assessed. The microbial flora on the labial surface of the teeth are different from those on the lingual surface,[48] and the lower vagina, upper vagina and cervix also differ in their microbial flora.[49] How this relates to the various niches in the female genital tract in pregnancy and to the human microbiome project as a whole is indicated in Figure 1.

MICROBIAL TAXONOMY

Cultivation-independent techniques require us to be aware of different levels of taxonomic classification. Taxonomy is the classification of living organisms into phylogenetic groups. Phylogenetic classifications arrange organisms into groups which reflect genetic similarity and evolutionary relatedness. A taxon is a group, or level of classification, and is hierarchical, whereby broad divisions are divided up into smaller divisions such as Domains, Kingdoms, Phyla, Classes, Orders, Families, Genera, and Species. Not every level is used in every discipline. The species is the basic unit of taxonomy and many molecular based studies use different terms such as “operational taxonomic units”, “taxons/taxa” or “phylotypes” instead of species, because the level of molecular diversity far outstrips that defined by existing microbiological or biochemical means. The human intestine demonstrates the greatest degree of diversity with over 400 phylotypes only 20% of which are cultivatable.[50]

MOLECULAR BASED TECHNIQUES

Many of the culture-independent studies focus on the detection of novel, previously uncultivated species[51-56] or are more concerned with the novel molecular techniques in themselves rather than for the microbiological information they provide.[57-60] In addition, theoretical and mathematical models have arisen which address new concepts such as "functional redundancy," "structural diversity," "interspecies interaction," "mutualism," "cheating," "the insurance hypothesis," "drivers" and "passengers".[61-64]

Bacterial DNA is extracted from samples, and is amplified using the polymerase chain reaction (PCR) using either universal or specific primers. This is not infallible because PCR inhibitors may be present at varying levels in individual clinical samples, and primers may preferentially amplify certain nucleic acids, and may not complement the entire bacterial kingdom.[65]

The commonest target for molecular identification of bacteria is the small ribosomal subunit of the 16S rRNA gene. The 16S rRNA gene is useful because it is present in all bacteria and has regions of conserved sequence which can be targeted by universal (sometimes referred to as bacterial-domain or broad-range primers) or specific primers, yet also has areas of heterogeneity which can be used to identify bacteria or to infer phylogenetic relationships.[66-71] If there is uncertainty with respect to which organisms might be present in a sample or if a broad diversity of organisms are anticipated, then many researchers will use universal primers.[53] If, on the other hand, an investigator knows what organisms to expect, or wishes to test for specific organisms then specific primers will be used.[72] Once the 16S rRNA gene has been sequenced, the variable regions can be used for species specific PCR in a qualitative or quantitative manner. The sequences obtained are aligned and compared with large databases of 16S rRNA sequences, although diversity for vaginal microbial flora is poorly represented in these databases compared to other sites like the gastro-intestinal tract.[54;60]

Cultivation-based studies have the disadvantage that they may fail to isolate or detect large numbers of fastidious micro-organisms, or identification tests may not be available. An unknown number of species which are identified purely by molecular methods are cultifiable, but have not been identified by cultivation methods because of a lack of phenotypic tools for the species, their lower relative titers, or inappropriate media (REF: Rabe LK, Antonio M, Austin MA, Stoner K, Pollard R, Petrina M, Leyland B, Chaiworapongsa T, Lamont RF, Hassan S, Romero R, and Hillier S. A 21st Century Description of the Vaginal Microflora of Pregnant Women. Proceedings of the Annual Meeting of the Infectious Diseases Society for Obstetrics and Gynecology, Santa Fe, New Mexico, USA, Aug 6th 2010.

This is an important distinction, since it shifts the focus of new research from developing novel culture media to using molecular tools to identify the under-characterized colonies that already grow in current formulations. This also restricts the development of their phenotypic profiles and hence the understanding of their roles in the whole population. In addition, this suggests that before the development of molecular techniques, culture-based assessments of the vaginal microbiome did not "miss" 80% of the species, but simply had no labels to impose on many colonies. As a result these organisms were included only as members belonging to a broad phenotypic or phylogenetic branch.

Cultivation-independent techniques may show greater diversity by overcoming cultivation problems with and identification of fastidious organisms, but are limited by their tendency to sample only the most prevalent bacteria in a community, such that low abundance or minority species are likely to be missed.[73] Since PCR amplification of DNA is a competitive

enzymatic reaction, the 16S rRNA templates in a sample are amplified in accordance with their abundance. As a result, the 16S rRNA genes of the numerically dominant population will be the most abundant amplicons following PCR. Populations that constitute <1% of the total community (yet may still be present in $>10^6$/g of vaginal fluid) may not be represented in such profiles, so this represents the threshold of detection. This being the case, despite their limitations, cultivation studies remain an important part of vaginal microbiology and will need to be used in combination with cultivation-independent techniques.[74] The development of the next generation of ultra-high throughput sequencing technologies (pyrosequencing) may remove an important quantitative barrier by increasing the number of reads from a gene or genome by many orders of magnitude in one experimental run.[75] The power of next generation sequencing, however, is not without limitations. Until recently, error rates using pyrosequencing were high enough to generate reading errors that generated false microdiversity at the species or subspecies levels. However, improvements in the technology in 3rd generation systems now produces sequencing with error rates similar to Sanger sequencing, which should minimize this is future experiments. Even this improvement does not overcome the best read lengths among these, (~ 350-400 bases). The variable domains of 16S rDNA, which allow best species discrimination, are spread out over >1kb, so only some can be captured in this run length. This limits discrimination to the genus level for some phylogenetic branches with minimal diversity at this locus.[76] This obstacle remains a stumbling block for most of the next generation sequencing methods, less so as read lengths are steadily improving via technical advancements. For an insight into the pros and cons of the various techniques and their limitations the reader is directed to three excellent reviews.[77-79]

NORMAL VAGINAL FLORA

Most studies, whether cultivation-dependent or independent, give the impression that the vaginal microbial flora is static, because most studies are carried out as a “snap-shot” in time and do not consider that vaginal microbial communities undergo shifts in their representation, abundance and virulence over time, and are affected by many factors.

Identification of Lactobacilli to the Species Level Using Molecular-based Techniques

Culture and microscopy of “normal” vaginal flora typically shows a predominance of *Lactobacillus* species which are believed to promote a healthy vaginal milieu by providing numerical dominance but also by producing lactic acid to maintain an acid environment that is inhospitable to many bacteria and is negatively correlated with BV.[80] Lactobacilli also produce hydrogen peroxide (H_2O_2),[81] antibiotic toxic hydroxyl radicals, bacteriocins,[82] and probiotics.[83] Prior to molecular-based techniques, lactobacilli were generally identified only to the genus level.

In 1892, Professor Albert Döderlein (1860-1941) published his definitive monograph in which he recorded that cultured organisms were a source of lactic acid which could inhibit the growth of pathogens *in vitro* and *in vivo*.[84] In 1928, Stanley Thomas identified Doderlein’s bacillus as *Lactobacillus acidophilus*, adding prophetically, that this was either a characteristic group of related species, or a species which underwent a remarkable transformation.[85] In 1980, in keeping with the observation of Thomas, a group of organisms previously collectively known as *L .acidophilus* was shown to be highly heterogeneous.[86] As a result, the group was divided into DNA homologous groups that could not be distinguished biochemically,[87] to form a number of separate species within the *L. acidophilus* complex (Table 1).[88-90] The closely related species within the *L. acidophilus* complex are difficult to differentiate by phenotypic methods which may account for the variation in species of lactobacilli found in different studies.[91-93]

Cultivation-based techniques, because they fail to detect fastidious organisms, underestimate the diversity of vaginal microbial flora, but because of deficiencies in the phenotypic identification of lactobacilli they overestimate the diversity of *Lactobacillus* species in the vagina.[94]

Some 20 years ago, using cultivation-based phenotypic techniques, Redondo-Lopez et al concluded that no two women were colonized by the same two *Lactobacillus* species.[92] Using cultivation-independent techniques this would appear now not to be the case and because of their significant role in health and disease, there has been much attention to the identification of lactobacilli using genotypic means.[51;65;91;95-98]

Lactobacillus iners: under-detected and under-appreciated

The existence of *Lactobacillus iners* was unknown prior to 1999 but is now known to play a significant role in vaginal microbial flora. Selective media such as Rogosa or MSR agar are normally used to culture lactobacilli, so the use of cultivation-based techniques, even those followed by molecular methods, will not detect *L. iners* because it only grows on blood agar.[90] Hence, some very important molecular based studies failed to isolate *L. iners* because Rogosa or MRS agar were used rather than blood agar.[51;95;99-101]

The first report of isolation of *L. iners* in a woman with a normal Nugent score was in 2002.[65] Cultivation-independent methods have identified *L. iners*, a lactic acid producing bacterium, as one of the organisms most frequently isolated from the vagina of healthy women.[62;65;97;98;102-104] In contrast to *L. crispatus*, which is rarely dominant in BV,[53] *L. iners* can be detected at high levels in most subjects with and without BV,[53;65;105;106] and in three studies it was the only *Lactobacillus* species detected in BV positive women.[52;53;105] It has been postulated that this may be because *L. iners* may be better adapted to the conditions associated with BV, i.e. the polymicrobial state of the vaginal flora and elevated pH.[105] Alternatively, the observations could result from relative resistance of *L. iners* to unknown factors that lead to the demise of other *Lactobacillus* species during the onset of BV, or to a relative lack of antagonism of *L. iners* to the BV-associated anaerobes, so that their dominance predisposes the individual to acquiring BV.

Numerical Supremacy of *Lactobacilli*

Over 120 species of *Lactobacillus* have been identified, and more than 20 species have been detected in the vagina. Using molecular-based techniques, we now know that healthy vaginal microflora does not contain high numbers of many different species of *Lactobacillus*. Rather, one or two lactobacilli from a range of three or four species (mainly *L.crispatus* and *L. iners* but also *L. jensenii* and *L. gasseri*) are dominant, whereas other species are rare, lower in titer, and tend to be novel phylotypes.[51;52;55;62;65;97;100;101;103;106-109]

In healthy Swedish women with a normal Nugent score,[110] 202 vaginal isolates were tested against 26 type and reference strains of *Lactobacillus*.[97] In 18/23 women, the vaginal flora was dominated by a single species of *Lactobacillus* and only five women had two different species or two different strains of the same species of *Lactobacillus*. The only species detected were *L. cripsatus, L. gasseri, L. iners, and L. jensenii*.[97] In a follow up study only one women was colonized by more than two *Lactobacillus* species, four were colonized by two different species and 17 were colonized by one single species.[98] Although this study was severely limited by the small number of colonies examined per patient (10 from blood, 100 from Rogosa agar), the exclusion of other species is in keeping with the theory of "competitive exclusion"[55] and the superior ability of *L. iners* and *L. crispatus* to compete with other bacteria for vaginal resources, a survival strategy known as "bacterial

interference".[111] Alternatively, the rare co-existence of multiple dominant species of *Lactobacillus* could be due to pre-emptive colonization by a particular species, or due to host factors that strongly influence which species are able to colonize the environment.

Cultivation-independent studies using molecular techniques have been published on different populations such as adolescent girls,[55] post menopausal women[65;112] and women from Belgium,[52;107;109] Brazil,[113] Bulgaria,[94] Canada,[57;65;112] China,[108] Germany,[106] Holland,[114] India,[115] Italy,[99] Japan,[100;116] Nigeria,[102;117] Sweden,[97;98] both Turkey and the USA,[118] the USA,[54;55;58;62;95;103] African-American women,[105] Caucasian and black North American women[103] and a multinational group of women from seven different countries.[51]

Racial variation and geographical area are important[51] and different racial groups within the same geographical region have significant differences in what is the dominant vaginal organism.[103] In most populations, *L. crispatus* is the commonest dominant isolate, [51-53;58;62;97;103;104] and white women are more likely to be dominated by *L. crispatus* and/or *L. jensenii* than any other species of *Lactobacillus*. [95] A number of genetic as well as environmental factors might explain at least in part this observation. Alternatively,diet might influence the *Lactobacillus* species resident in the gastro-intestinal tract and hence the vagina since the lactobacilli of the gut varies between Japanese and Western subjects.[119-121]

Production of H_2O_2 by Lactobacilli and the Association with Bacterial Vaginosis

Lactobacilli differ in their ability to produce H_2O_2 and a reduction in the prevalence and concentration of H_2O_2 producing bacteria is associated with the development of BV and vaginal infections.[81;122] With the introduction of molecular based techniques, it is now possible to relate the production of H_2O_2 to individual species or strains of the same species of *Lactobacillus* rather than the genus as a whole. Three studies using molecular based techniques have addressed the production of H_2O_2 by lactobacilli.[95;100;101] In Japanese women who did not have BV, *L.crispatus* and *L. gasseri* were found in the vagina of 52.7% and 20.8% of women respectively. *L. jensenii* was not detected, All strains of *L. crispatus* were strongly positive for H_2O_2, whereas 41% and 59% of *L. gasseri* strains were strongly and weakly positive for H_2O_2 respectively.[100]

Antonio et al (1999) demonstrated that *Lactobacillus* species detected among 302 women with and without BV differed in their ability to produce H_2O_2. *L. crispatus* and *L. jensenii* were found to colonize 32% and 23 % of women respectively and 95% and 94% of their strains, respectively, were shown to produce H_2O_2. In contrast, *L. gasseri* and *L. iners* colonized 5% and 15% of women respectively and only 71% and 9% of their strains respectively produced H_2O_2. Not surprisingly, BV was present in 9% and 7% of women colonized by *L. crispatus* and *L. jensenii* respectively, and in 43% and 36% of women colonized by *L. gasseri* and *L. iners* respectively. Of the women without BV by Nugent score,[110] 16% had no lactobacilli present, and none of the women colonized by *L. crispatus* and *L. jensenii* had BV; however, the latter argument is circular, since the *Lactobacillus* morphotype is part of the Nugent score. The association between *L. gasseri* and BV has been confirmed in a study of homosexual women. Detection of *L. gasseri* was associated with a 4.2 fold increased risk of BV,[123] and attributed to a higher rectal colonization by *L. gasseri* and sexual practices which increase the risk of vaginal colonization from the rectum.[124]

Using culture-based techniques Eschenbach et al[122] and McGroarty et al[125] found that 100% of *L. jensenii* produced H_2O_2, yet Nagy et al1[26] found only 46%. Similarly, with *L. acidophilus* the range of species which produced H_2O_2 varied from 43% to 77%.[122;125;126] This may have been due to the inability of biochemical assays to differentiate between the

species belonging to the *L. acidophilus* complex. However, this led Nagy et al to conclude, that the ability of lactobacilli to produce H_2O_2 was associated with the origin of strain (whether from women with or without BV) rather than the *Lactobacillus* strain itself.[126] In light of the findings of molecular-based techniques, and the current ability to identify H_2O_2 producing lactobacilli to the species level,[95] we might alternatively conclude that it is whether or not the strain/species of *Lactobacillus* produces H_2O_2 that dictates whether BV is present or absent. However, given that H_2O_2-producing *L. gasseri* are found in BV patients, albeit at lower incidence, one might also argue that in vitro production of H_2O_2 is only a biomarker of a protective species of *Lactobacillus*, not an active factor in limiting the growth of vaginal anaerobes. Indeed, it is not clear whether bacterial H_2O_2 production is active in the microaerobic to anaerobic vaginal flora. Other factors may include the extent to which a species can dominate numerically over its competitors, e.g. 95% versus 99.99%.

Healthy vaginal flora not dominated by lactobacilli

Using culture-independent techniques several investigators have demonstrated that a significant proportion (7-33%) of healthy women lack appreciable numbers of *Lactobacillus* species in the vagina[52;58;62;102;103] which may be replaced by other lactic acid producing bacteria such as *Atopobium vaginae, Megasphaera* and *Leptotrichia* species.[62;103] Although the structure of the communities may differ between populations, health can be maintained provided the function of these communities i.e. the production of lactic acid, continues.[55;62] Consequently, the absence of lactobacilli or the presence of certain organisms such as *G.vaginalis*, or species of *Peptostreptococcus, Prevotella, Pseudomonas*, and/or *Streptococcus*, does not constitute an abnormal state.[58] This issue is still unclear, however, because the studies do not address whether some proportion of “healthy” women are patients in transition to or from BV, or whether they have asymptomatic BV, ie. abnormal flora but no symptoms due to genetic or other factors. Indeed, a recent molecular study further confuses the issue by pointing out that *G. vaginalis* may produce transient dominance in healthy women as a result of perturbations such as increase in pH, during menstruation.[127]

Vaginal Probiotic Lactobacilli

The first example of vaginal probiotics use we could detect was that of Stanley Thomas in 1928 following his observation that *Lactobacilli* were absent in the presence of gonococci. He reported on two experiments, one *in-vitro* and the other *in vivo* in which addition of a thin layer of whey broth from a culture of *L. acidophilus* demonstrated eradication of *Neisseria gonorrhoeae*.[85]

Exogenous strains of *Lactobacilli* have been suggested as a means of establishing or re-establishing normal vaginal flora. *L. fermentum* and *L. rhamnosi* probiotic strains have been used with poor results in urogenital infection and this may be because they are not normally prevalent in the vagina.[104;128;129] In contrast, *L. crispatus* might be a better choice because it is commonly found to be numerically dominant in the healthy vagina and 95% of the strains produce H_2O_2.[95] *L. crispatus* may have a superior capacity to persist in the vagina[130] and strains of probiotic *L.crispatus* CTV-05 have been demonstrated to have high mean adherence to vaginal epithelial cells *in vitro* [131] and established vaginal colonization in 7 of 9 women when administered vaginally.[132] Combined vaginal and rectal colonization by H_2O_2 producing *Lactobacilli* is associated with a four-fold decrease in the incidence of BV.[131]

Using molecular based techniques in a double blind, randomized, single centre study of 90 non-pregnant, sexually active women, who were free from genital infection, and had a normal Nugent score, the ability of the probiotic *L. crispatus* CTV-05 to establish vaginal colonization was studied.[133] The study demonstrated that *L. crispatus* CTV-05 established

vaginal colonization at one or more follow-up visits in 69% of women overall and 90% of those not already colonized with *L. crispatus*. Of women who were never colonized with *L. crispatus* CTV-05, 85% were already colonized by endogenous *L. crispatus* at enrollment. The authors saw this as self regulatory rather than a shortcoming of the probiotic, and the dosage regimen did not result in overgrowth of *Lactobacilli* since there seemed to be a physiological adjustment to around 10^6 or 10^8 cfu/mL of vaginal fluid. They also recommended that future research should concentrate on *Lactobacilli* which are prevalent in the vagina[133] rather than species such as *L. fermentum* and *L. rhamnosus*.

ABNORMAL VAGINAL FLORA

Abnormal vaginal flora may occur because of a sexually transmitted infection (STI) eg trichononiasis, colonization by an organism which is not part of the normal vaginal community e.g. *Streptococcus pneumoniae, Haemophilus influenzae*, or *Listeria monocytogenes*, or by overgrowth or increased virulence of an organism that is a constituent part of normal vaginal flora e.g. *Escherichia coli*. Alterations in vaginal flora do not necessarily imply disease or result in symptoms. Disease results from interplay between microbial virulence, numerical dominance, and the innate and adaptive immune response of the host.[134] The most common disorder of vaginal flora is BV. BV is a polymicrobial condition, characterized by a decrease in the quality or quantity of *Lactobacilli* and a one thousand-fold increase in the number of other organismsas determined by cultivation-dependent techniques, particularly anaerobes *Mycoplasma hominis, Gardnerella vaginalis* and *Mobiluncus* species. The prevalence of BV in pregnancy in the USA is 1,080,000 cases annually.[135] In pregnancy, BV has been associated with early,[11] and late miscarriage,[12;136] recurrent abortion,[12] post abortal sepsis,[10] post partum endometritis,[30] and preterm birth.[12;21;136-138]

BV and HIV

BV is also associated with acquisition of HIV.[5-9] Healthy lactic acid producing vaginal flora acts as a barrier against acquisition of HIV[139;140] and is negatively correlated with BV[80] which acts as a co-factor for HIV and conversion to seropositivity.[5-9] HIV infected women with BV have higher levels of HIV viral load in genital secretions than do HIV infected women without BV.[6;141;142] BV is also associated with increased susceptibility to other STIs including herpes simplex virus -2, gonorrhoea, *Trichomonas vaginalis* and *Chlamydia trachomatis*.[141;143-145] Molecular based studies suggest a trend towards increased diversity in the microbiome of HIV positive women with BV compared to those HIV positive women who do not have BV suggesting that HIV infection *per se* is associated with changes in the diversity of genital microbiota.[146]

Individual bacteria as a cause of BV

Many of the models necessary to demonstrate that bacteria function as mono-etiological agents require a change in the micro-environment before an infectious event is observed. Infective peritonitis is easier to induce in experimental animals if blood is instilled into the peritoneal cavity, experimental gangrene develops better if calcium chloride is implanted into muscle along with *Clostridium* species, and rodents will not develop vaginal candidiasis without the addition of estrogen.[42] In the definitive paper of Gardner and Dukes (1955), the instillation of pure cultures *Haemophilus vaginalis* (now known as *Gardnerella vaginalis)* only successfully induced vaginal colonization in one of 13 volunteers.[147] In contrast, 11 of 15 volunteers were successfully inoculated when the vaginal secretions of donors (screened for other genital tract infections) were instilled into the vagina. This supports the later view of others, that since vaginal secretions from donors were much more successful at causing

disease than pure cultures, *G. vaginalis* probably acts synergistically with other organisms to cause BV.[148]

Atopobium vaginae: under-detected and under-appreciated

The genus *Atopobium* lies within the family *Coriobacteriaceae* and forms a distinct branch within the phylum *Actinomycetes*.[149] Following sequence analysis, three species formally designated *Lactobacillus minutus*,[150] *Lactobacillus rimae*,[151] and *Streptococcus parvulus*[152] within the lactic acid producing group of bacteria,[153] have been reclassified as genus *Atopobium*. In 1999, an organism similar but not identical to these three species was isolated from the vagina of a healthy woman in Sweden and the organism was named *Atopobium vaginae*.[154] Since that time, using molecular-based techniques, *A. vaginae* has frequently been detected in the vagina and is found much more commonly in women with BV than in those with normal flora.[52-55;59;62;72;103;105-107;109;112;113;155-160] In addition to producing lactic acid,[161;162] some species of *Atopobium* have peptidyl peptidase activity and produce significant amounts of ammonia in other environments[163-165] where sugars are a scarce source of energy. This may be why *A. vaginae* is found more often in the vagina of postmenopausal women, not on hormone replacement therapy (HRT) compared to those who are taking HRT.[112] *Prevotella bivia* (formerly *Bacteroides bivia*) also produces ammonia which is known to act as a substrate to promote the growth of *G. vaginalis*.[166] *A. vaginae* is strictly anaerobic and *in-vitro* is very sensitive to clindamycin[157] but highly resistant to nitro-imidazoles such as metronidazole[157] and secnidazole.[156]

High Diversity of flora in BV compared to normal flora

Using various molecular-based techniques and usually Amsel clinical criteria[167] or Nugent score[110] to classify normal or abnormal flora, a number of studies have demonstrated a high diversity of organisms in women with BV compared to women with normal flora. Collectively, these studies demonstrate the presence of novel bacterial species previously unidentified using cultivation-dependent techniques.[51-56] They have also demonstrated that many of these organisms have specificity for BV and that the number of phylotypes found in association with BV is statistically significantly greater than the number detected in the presence of intermediate flora (a distinct entity in its own right)[168;169] or normal flora.[49;52-54;56;65;105-107;109;157] This statistic is largely due to the extreme dominance of lactobacilli in healthy women, which makes detection of other species unlikely, even when they are present 100,000 or more cells per sample.

Many of these organisms will be unfamiliar to clinicians (Table 2) though for many of them, there is evidence of disease association. The renamed *Atopobium parvulum*, *Atopobium minitum* and *Atopobium rimae* have been associated with dental abscesses and oral infections,[151;162;165;170;171] tubo-ovarian abscesses,[172] and abdominal wound infection, supporting the view that these organisms may be pathogenic to the host. *Leptotrichia sanguinegens/amnionii* has been reported in association with post-partum endometritis, adnexal masses and fetal death,[173-175] and has been detected in the amniotic fluid of women with PTL, PPROM and preeclampsia.[176-178] Also, in a study of 45 women with salpingitis and 44 controls (women seeking tubal ligation), bacterial 16S rRNA sequences were found in the fallopian tube specimens of 24% of cases and in no controls. Bacteria phylotypes closely related to *Leptotrichia* species and *A.vaginae* were among those identified in cases.[179] In addition, *Dialister pneumosintes* was found as the sole agent in blood culture from a women with suppurative postpartum ovarian thrombosis.[180]

In summary, these studies have demonstrated that different subjects with BV have different microbial profiles indicating heterogeneity in the composition of bacterial taxa in women with BV. Women without BV had bacterial communities dominated by *Lactobacillus*

species accounting for 86% of all sequences. In contrast, women with BV did not possess a single dominant phylotype but contained a diverse array of vaginal bacteria, often at relative low abundance.

Molecular-based tools for diagnosis of BV

BV can be diagnosed clinically, or using composite clinical criteria,[167] microscopically,[110;181-186] enzymatically,[187-190] chromatographically[191;192] or using qualitative or semi-quantative culture methods.[19] Currently, the gold standard is Nugent score[110] but the number of methods testifies to the fact that no single test is ideal and that they can all provide false positive and false negative results. Findings from molecular-based studies are now highlighting possible explanations for why diagnosis by microscopy may be inconsistent and why molecular methods may replace them.

One of the three organisms quantified as part of the Nugent score is *Mobiluncus*. Several cloning and sequencing studies have identified *Mobiluncus* only rarely.[53;58;106] Fluorescence in situ hybridization (FISH) technology has demonstrated that BV associated bacterium (BVAB)-1 has a curved rod morphology,[53] similar to *Mobiluncus* morphotypes, and it is possible that with microscopic examination of vaginal smears, *Mobiluncus* species may have been overrepresented and mistaken for BVAB-1.[62;193] Alternatively, since species specific PCR agrees with the Nugent score *Mobiluncus* may be missed in universal PCR studies because it frequently falls below a threshold titre where it can be detected.[78]

Zhou et al ([2004]) observed that the urea produced by *Atopobium* species was associated with halitosis,[164] and that similar species of *Megasphaera* caused beer spoilage by turbidity, off-flavors and off-colors.[194;195] They concluded that if two genera associated with malodorous metabolites can be found in the vagina of healthy women, and amines can be found in women without BV, then diagnostic techniques to diagnose BV, based on upon amine production and odor formation[167;189;192] may need to be amended.[62]

Microscopically, *Atopobium* species are gram positive, elliptical cocci or rod-shaped organisms that occur, singly, in pairs or short chains. The variable cell morphology of *Atopobium* renders it well camouflaged among the mixture of other species present in bacterial communities where the Nugent score is ≥4. *A. vaginae* is fastidious, grows anaerobically and forms small pin-head colonies on culture that are easily missed.[154] Although phylogenetically different from other lactic acid producing bacteria, they are not phenotypically exceptional and it is not difficult to see why the significance of this organism based on culture, microscopy and phenotype may be overlooked and under-appreciated.

Using species specific primers, the relationship between five fastidious organisms associated with BV, were compared with BV diagnosed by Amsel and/or Nugent scores, and also with the individual clinical criteria of Amsel.[196] The two biovars of *Ureaplasma urealyticum* (*U. parvum* and *U. urealyticum* – biovar 2) were associated with vaginal discharge and raised pH, but not with BV by either Amsel or Nugent criteria or any of the individual Amsel clinical criteria. In contrast, with *Leptotrichia sanguinegens/amnionii, A.vaginae* and BVAB-1, elevated pH > 4.5 was a universal feature and they were all associated with BV by both Amsel and Nugent criteria, and with the finding of >20% of epithelial cells as clue cells, a feature already reported.[53] A positive test for amine odor on addition of 10% solution of potassium hydroxide was significantly more likely in women testing positive for BVAB-1. Douching is a recognized risk factor for BV[197] and the detection of *Leptotrichia* and *A. vaginae* was three times more likely and BVAB-1 twice as likely when women reported douching.[196]

Fredricks et al, in two linked studies noted that some organisms or combination of organisms had high sensitivities or specificities for the diagnosis of BV using Amsel criteria[53;72] and Nugent score.[53] (Table3).Using quantitative real time PCR, Menard et al firstly examined qualitatively the association of individual organisms with BV diagnosed by Nugent score. To optimize the molecular methods for routine practice, an adjusted quantification was made creating a threshold of DNA copies/mL for lactobacilli and several organisms known to be associated with BV (Table 4). At a threshold of $\geq 10^8$ DNA copies/mL, *Lactobacillus* species was predictive of normal flora (Sensitivity 44%, Specificity 100%). BVAB-1, BVAB-2 and BVAB-3 alone or in combination had high specificity for BV diagnosed by Amsel criteria.

The combination of *A. vaginae* and *G. vaginalis* for the diagnosis of BV

Since *A. vaginae* and *G. vaginalis* are frequently detected in association with BV a number of authors using molecular-based techniques have examined the possibility of combining these two organisms as a means of diagnosing BV.[52;53;59;112;155;159;160] Using DNA quantitation, 19/20 BV samples had either a DNA level for *A. vaginae* $\geq 10^8$ copies/mL or *G.vaginalis* $\geq 10^9$ copies/mL and 9/20 had both. The combination of an *A.vaginae* DNA level $\geq 10^8$ copies/mL and a *G.vaginalis* DNA level $\geq 10^9$ copies/mL demonstrated the best predictive criteria for the diagnosis of BV with excellent sensitivity (95%) specificity (99%) negative predictive value (NPV) (99%) and positive predictive value (PPV) (95%).[159] When the quality and reproducibility of this combination was applied prospectively for validation of the Nugent score in 56 pregnant women the NPV was 96% and the PPV was 99%.[159]

Culture-Independent Techniques to Assess the Treatment of Recurrent, Persistent or Resistant BV

Cure of BV or improvement in symptoms following recommended treatments with metronidazole or clindamycin[135] reaches 83-94% by seven to 21 days.[198;199] While the short term treatment response is acceptable, BV persists or recurs in 11-29% of women at one month,[198;200;201] 30% of patients relapse within three months and recurrence rates may be more than 50% within a year.[155;202-204] Only 48% of women will be colonized by H_2O_2 producing *Lactobacilli* 70-90 days after treatment with either clindamycin or metronidazole.[201;205]

A number of molecular-based studies[53;105;155;206;207] have addressed the problems of recurrent or persistent BV over time, and why some women with BV may be resistant to cure. A consistent finding in these studies as women lapse in and out of being BV positive and BV negative is the stability of vaginal flora requiring few phylotypes and *Lactobacillus* dominance with *L.crispatus* and/or *L. jensenii* in women who remain or become BV negative. In contrast, those who become BV positive are commonly colonized by *L. iners* with many other non-*Lactobacillus* species present.[53;105] Three studies have addressed the problem of persistent or recurrent BV using culture–independent techniques.[155;206;207] Women with BV were appropriately treated and followed up in a large, prospective, cross-sectional cohort study over a 12 month period. *A. vaginae* was detected in 75% and *G. vaginalis* in 100% of women with recurrent BV. Women in whom both organisms were detected had higher rates of recurrence of BV (83%) compared to women with *G. vaginalis* alone (38%) ($p<0.001$). Of relevance is the fact that >90% of a biofilm indentified on vaginal epithelial cells of women with BV was composed of *A. vaginae* and *G. vaginalis*.[208;209] The biofilm may have interfered with treatment, but the authors were unable to determine whether recurrence was due to inadequate treatment and undetectably low levels of residual organisms after treatment (i.e. relapse), or re-infection from sexual partners, which they thought was feasible, or disruption of normal flora from other exogenous factors.[155]

In an isolated study, several bacterial species were detected, none of which are commonly associated with BV. Organisms normally associated with BV were not detected. This atypical form of BV might explain why conventional therapy did not work and may indicate that other anti-microbial therapies may have been more effective.[206] Finally, using species specific 16S rDNA PCR assays targeting 17 different BV associated bacteria in 131 women with BV, the vaginal microbiome was sampled pre-treatment and one month after for test of cure. Treatment was with a five day course of intravaginal metronidazole gel. At one month after treatment, BV was still present in 26% of women. The baseline detection of BVAB -1, BVAB -2, and BVAB -3, *Peptoniphilus lacrimalis* or *Megasphaera* phylotype was significantly associated with persistence of BV at test of cure. The authors concluded that pretreatment vaginal microbiology at diagnosis might define the risk of antibiotic failure.[207] We anticipate that these correlations will become clearer and more meaningful when studies are repeated with quantitative PCR instead of simply using detection / incidence.

Culture-independent studies in pregnancy

Culture independent techniques have been used to measure prevalence, diversity and abundance of organisms particularly ureaplasmas[210] in amniotic fluid in association with suspected cervical insufficiency,[211] preterm labor,[176;212] PPROM,[178;213] babies small for gestational age,[214] preeclampsia[177] and the potential for bacteria from the oral cavity to colonize amniotic fluid.[215] However, apart from combining pregnant women with non-pregnant women to swell sample numbers,[52;160] the information with respect to the vaginal microbiome in pregnant women is limited particularly with respect to the outcome of pregnancy, especially preterm birth.

Using species specific primers, Wilks et al,[101] quantified the production of H_2O_2 by lactobacilli from swabs taken at 20 weeks gestation from the vagina of 73 women considered to be at high risk of preterm birth according to the Creasy criteria.[216] The amounts of H_2O_2 production varied between species of *Lactobacillus*. The presence of lactobacilli producing high levels of H_2O_2 was associated with a reduced incidence of BV at 20 weeks gestation and subsequent chorioamnionitis. The authors postulated that H_2O_2-producing lactobacilli reduced the incidence of ascending genital tract colonization in pregnancy which leads to infection and preterm birth.[101] Unfortunately the role of *L. iners*, was not tested, because MRS agar culture medium was used in the study.[90]

In pregnant Japanese women, Tamrakar et al[116] largely confirmed the findings of Fredricks et al 2005[53] in non-pregnant women. The prevalence of *L.crispatus, L. jensenii*, and *L. gasseri* was significantly higher, while that of BVAB-2, *Megasphaera, Leptotrichia* and *Eggerthella*-like bacterium were significantly lower in women with a normal Nugent score, compared to those with BV. The prevalence of *L. iners* did not differ between the these groups, and women with *L. iners* were more likely to be colonized by BVAB-2, *Megasphaera*, *Leptotrichia* and *Eggerthella*-like bacterium.[116]

In a longitudinal study of 100 pregnant women, vaginal swabs were obtained at mean gestational ages of 8.6, 21.2, and 32.4 weeks respectively.[217] In first trimester, 77 women had normal or *Lactobacillus* dominated flora, 13 of whom developed abnormal flora in the second or third trimester. When first trimester normal flora was dominated by *L. gasseri* or *L. iners* there was a 10-fold risk of conversion to abnormal flora. In contrast, normal flora comprising *L. crispatus* had a five-fold decreased risk of conversion to abnormal flora.[217] This may be because *L. gasseri* and *L. iners* only produce H_2O_2 in a small percentage of strains.[95;131]

CONCLUSIONS

Molecular based techniques provide important new information about vaginal microbial flora and permit identification of previously under-detected and hence under-appreciated organisms such as *L. iners* and *A. vaginae*. Molecular based techniques are not without their problems, and will not replace culture based techniques, but, when used in combination, add greatly to our understanding of vaginal flora. In the majority of circumstances, normal vaginal flora is dominated by *Lactobacillus* species. In the absence of lactobacilli, normality can be maintained by other, more fastidious lactic acid producing bacteria. In keeping with the theories of "competitive exclusion" and "bacterial interference", when lactobacilli dominate the vaginal flora, culture-independent techniques have demonstrated that the healthy vagina is usually colonized by only one or two dominant *Lactobacillus* species, mainly from *L. crispatus, L. iners, L. jensenii* and *L. gasseri*. The dominant *Lactobacillus* species may differ racially or geographically, but the principle of numerical dominance persists, and may be an important defense mechanism. Without molecular based techniques, phenotypic identification of lactobacilli is difficult and so is normally only carried out to the genus level. By being able to identify lactobacilli to the species level, we should be better able to understand the role of different species of *Lactobacillus*, particularly with respect to their ability to produce H_2O_2 and to function as a probiotic.

Molecular-based techniques indicate that there is a far greater diversity of microorganisms associated with BV than has been evident from cultivation-dependant techniques. These diverse organisms accumulate to form different communities or profiles which make it likely that BV is not a single entity, but a syndrome of variable composition which cause a variety of symptoms, different phenotypical outcomes, and may result in variable responses to different antibiotic regimens. Some organisms or combinations of organisms have a high specificity for BV such that in future using molecular quantification, we may better diagnose each sub-type of BV and tailor treatment appropriately. The information with respect to the vaginal microbiome in pregnant women is limited particularly with respect to preterm birth. This gap is striking, given the importance of preterm birth problem worldwide, and must become a research and funding priority. By better understanding the vaginal microbiome during pregnancy, we may be able to predict and prevent some of the great obstetric syndromes like PPROM, preterm labor and preterm birth which is associated with infection and significant infant mortality and morbidity. This better understanding is imminent, with the onset of studies using much more powerful sequencing technologies.

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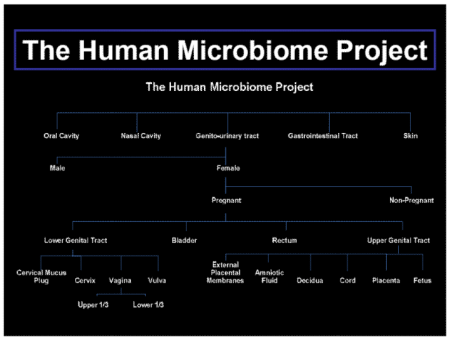

Figure 1.
The Human Microbiome Project

Table 1

Obligately Homofermentative Species Within the *Lactobacillus acidophilus* Complex.

Lactobacillus acidophilus
Lactobacillus amylolyticus
Lactobacillus amylovorus
Lactobacillus crispatus
Lactobacillus gallinarium
Lactobacillus gasseri
Lactobacillus iners
Lactobacillus jensenii
Lactobacillus johnsoni

Table 2

Previously unfamiliar vaginal organisms identified by molecular based techniques

Atopobium vaginae,
BV associated bacteria [BVAB]-1, BVAB-2, and BVAB-3 in the order *Clostridiales*,
Megasphaera spp
Leptotrichia spp
Dialister spp
Chloroflexi spp
Olsenella spp
Streptobacillus spp
Shuttleworthia spp
Porphyromonas asaccharolytica

A bacterium distantly related to *Eggerthella hongkongensis* (92% sequence similarity)

Table 3

The sensitivities and specificities for individual or combinations of organisms for the diagnosis of BV using Amsel criteria or Nugent score

Study	Diagnosis of BV	Organism	Sensitivity	Specificity
Frederick et al[53]	Amsel	BVAB-1	40.7	97.8
Frederick et al[53]	Amsel	BVAB-2	88.9	95.7
Frederick et al[53]	Amsel	BVAB-3	40.7	97.8
Frederick et al[53]	Amsel	*G.vaginalis*	100	41.3
Frederick et al[53]	Amsel	BVAB-1 and BVAB-3	33.3	100
Frederick et al[53]	Amsel	BVAB-2 or *Megasphaera*	100	91.3
Fredericks et al[72]	Amsel	Either *Megasphaera* or BVAB-1, BVAB-2, or BVAB-3	99	89
Fredericks et al[72]	Nugent	Either *Megasphaera* or BVAB-1, BVAB-2, or BVAB-3	95.9	95.7

BVAB = bacterial vaginosis associated bacteria

Table 4

Quantification of vaginal organisms for the production of BV using Nugent score[159]

Organism	Threshold Quantification (DNA copies/mL)	Sensitivity	Specificity	NPV	PPV	ROC AUC
A. vaginae	$\geq 10^8$	90	99	99	95	0.964
G. vaginalis	$\geq 10^9$	50	100	94	100	0.946
M. curtisii	$\geq 10^5$	45	100	-	-	0.798
M. hominis	$\geq 10^6$	30	98	-	-	0.691

NPV = Negative predictive value
PPV = Positive predictive value
ROC = Receiver operating characteristic
AUC = Area under the curve (The closer the AUC comes is to 1.0, the better the bacterial count predicts BV)

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