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Exploring a Road Map to Counter Misconceptions About the Cervicovaginal Microbiome and Disease

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Abstract

Urogenital diseases, especially infection and cancer, are major causes of death and morbidity in females. Yet, millions of women in the developing world have no access to basic urogynecological care, and the diagnosis and treatment of widespread aberrant bacterial conditions (bacterial vaginosis [BV] and aerobic vaginitis [AV]) remain suboptimal the world over. Samples from women living in resource-disadvantaged and developed countries have been analyzed by high-throughput sequencing to reveal the diversity of bacteria in the vagina, how rapidly the bacterial population fluctuates over time, and how rapidly the switch occurs between healthy and aberrant conditions. Unfortunately, clinical diagnostic methods are inefficient and too often outdated therapies are administered. The net result is suboptimal care and recurrent disease that adversely affects the quality of life. This viewpoint outlines a scientific and translational road map designed to improve the cervicovaginal health and treatment of disease. This comprises (1) improving education of women and physicians on the vaginal microbiota; (2) having agencies target funding for research to improve diagnosis and test new therapies; and (3) making sure that new approaches are accessible in developing countries, empowering to women, and are acceptable and appropriate for different populations.

Keywords

cervicovagina, bacteria, vaginosis, diagnosis, gardnerella, lactobacillus, Nugent, Amsel, microbiome

Which Bacteria are Associated With Health and Disease?

A series of high-throughput sequencing studies have recovered over 250 different bacterial taxonomic units from the vagina of women of differing ages, health status, and country of origin.[1-4] Longitudinal sampling has revealed that communities sometimes change markedly over short periods of time, while others are relatively stable, independent of the vaginal community compositions. Five types of temporal patterns of variation have been observed, including stable communities that do not contain any *Lactobacillus* sp. Of those healthy women dominated by *Lactobacillus*, the species *L crispatus*, *L iners*, *L jensenii*, and *L gasseri* are most commonly found.[5,6] This raises the question of what is normal? Further studies suggest some degree of variation in abundance profiles of bacterial types depending upon race,[7] but essentially there is tremendous commonality in the species of bacteria and community types detected across populations around the world, even though their frequencies can differ.

The acquisition of sequence data might appear to some clinicians to be far removed from everyday practice, but those wishing to obtain such data will be interested in the process.

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The articles cited in this review[1-7] and others are not universal as there is variation in the physiological collection site, in whether the samples were self-collected versus collected by a caregiver, or in the methods to determine which organisms are present. (DNA extraction, which primers were used, which sequencing method). Yet, collectively there is little disagreement about the findings, namely the dominance of lactobacilli in most healthy cases and its depletion in disease (as will be discussed). In essence, when bacterial DNA is extracted from the vagina and surrounding areas, and then sequenced and compared to databases of known bacterial sequences to determine its species it provides a road map of the organisms and their relative abundance present at that time and location. If these organisms could be cultured and quantified, the clinicians would be better informed as to how optimally to manage the patient in terms of infection; but many of these bacteria are not easy to culture. If the microbiota profiles (based upon DNA sequencing) are obtainable, preferably from several samplings over a given timeframe like 1 week or month, it becomes easier to understand what, if any, medical intervention is needed, based upon the patient's history and presentation.

The importance of having more than a single sample is highlighted by the finding that a healthy microbiota can switch to an aberrant one rapidly within days and in a portion of women it can also resolve quickly and without treatment.[8,9] These fluctuations and the fact that many women are asymptomatic with what would be called an aberrant vaginal microbiota based upon microscopic analysis, increase confusion over what is meant by a healthy or bacterial vaginosis (BV) or aerobic vaginitis (AV) condition, independently from risk of acquisition of secondary diseases. There are some similarities to *Escherichia coli* and urinary tract infections (UTIs), where pathogens with the armamentarium to cause symptomatic illness can be found in the bladder of asymptomatic women.[10] Likewise, around 25% of UTI cases spontaneously resolve.[11] If the bacteria do not produce symptoms and rapidly disappear, are they pathogens at that time? Does their presence constitute infection? Some might say yes, because the organisms have virulence factors and their presence meets the general definition of infection: "invasion by and multiplication of pathogenic microorganisms in a bodily part or tissue, which may produce subsequent tissue injury and progress to overt disease through a variety of cellular or toxic mechanisms." Some clinicians might say no, because the condition does not warrant treatment. For BV, it is not so much that the organisms regarded as pathogens, namely *Gardnerella vaginalis*, *Atopobium vaginae*, *Prevotella bivia*, some *Clostridiales* species, including 3 newly recognized bacteria provisionally named bacterial vaginosis–associated bacteria (BVAB) 1, 2, and 3,[12] are only present at appreciable levels when the microbiota is aberrant. Rather, it is their abundance that appears to diminish significantly when the vaginal status returns to normal. In addition, gross taxonomic classification is insufficient since small difference in genomic content between strains could alter pathogenic capability.

In the case of AV, a condition associated with red inflammation, yellow discharge, and vaginal dyspareunia, spontaneous resolution has not been investigated, and with causative or associated agents being *E coli*, *Staphylococcus aureus*, and Group B streptococci,[13] and parabasal epithelial cells, and/or positive for increased numbers and activity of vaginal leucocytes visible under the microscope,[14] this would be classified as an infectious condition. concerningly, AV is seldom diagnosed or even looked for during the examination of women having a routine examination or reporting symptoms and signs of vaginal discomfort, and wet mount examinations for accurate diagnosis of AV are almost never performed outside of specialized centers. Yet, the combination of the assessment of the microbiota with the associated host-response findings offers unseen opportunities to better understand the pathogenesis and potential complications of the disease and facilitates tailoring of treatment.

Therefore, the determination of the status of a woman's vagina with regard to its bacterial content is not universally performed, and likewise the diagnosis of an infectious condition is often arbitrarily decided based upon methods that are outdated and fairly inaccurate, as will be discussed in the next section. The single factor that is apparent is that lactobacilli are the dominant organism in the vast majority of healthy vaginas, and their depletion in favor of what is believed to be pathogenic species could correspond to an aberrant condition. As it is unclear what BV is, how best to diagnose and treat it, and whether it coincides or is separate from AV, it seems a good time to reevaluate what is meant by vaginal health and the risk of infection posed by various microbiota profiles.

More Problems With Diagnosis and Therapy

If we cannot define normal then how can we determine whether someone has an aberrant microbiota? In essence we do not. First, because high-throughput microbiota sequencing is not yet part of the diagnosis of disease or routine health check; and second, without symptoms and signs of disease patients would rarely be treated, unless an aberrant profile (as yet undefined) puts them at risk of another disease. This of course assumes that clinical checkups occur (not the case in most developing countries)[15] and that they are informative. In a recent study, a small proportion of women reported vaginal malodor (8%) but 42% had odor when a clinician used the 10% KOH test (Boon et al, unpublished data). This is part of the Amsel test first established in 1983,[16] 28 years after *Gardnerella vaginalis* was reported to be the cause of BV.[17] The remainder of the assessment includes vaginal pH elevated above 4.5, the presence of "clue" cells seen under the microscope, and a thin milky vaginal discharge that is different in form from normal discharge and that associated with vulvovaginal candidiasis. However, the specificity of abnormal vaginal discharge is not higher than 49%,[18] and vaginal pH in normal asymptomatic women ranges from 4.5 to 4.8 in different ethnic groups.[19]

Other attempts have been made to differentiate an aberrant from healthy vagina, based upon Gram staining of vaginal smears and enumerating the number of gram-positive rods,

Table 1. A comparison of known genomes of vaginal lactobacilli with *S. aureus*[a]

			# proteins in each organism containing adhesin-related domains				
	Pfam ID	Annotation	*L. reuteri* RC-14	*L. jensenii* 1153	*L. rhamnosus* GR-1	*L. crispatus* ST1	*S. aureus* Newman
General adhesion	PF01468	GA module		2			2
	PF02216	B domain					2
	PF03642	MAP domain					3
	PF07501	G5 domain					1
	PF01473	Putative cell wall–binding repeat	1				
Ig	PF02368	Bacterial Ig-like domain (group 2)					
	PF07523	Bacterial Ig-like domain (group 3)		1	3	3	1
Mucus	PF06458	MucBP domain	3	8	1	4	
Collagen	PF05738	Cna protein B-type domain		2	5		3
	PF05737	Collagen binding domain					
	PF01391	Collagen triple helix repeat (20 copies)			1		3
	PF02352	Decorin binding protein		1			
Fibronectin / fibrinogen	PF02986	Fibronectin binding repeat					
	PF05833	Fibronectin-binding protein A N-terminus (FbpA)	1		1	1	1
	PF10425	C-terminus of bacterial fibrinogen-binding adhesin	1				7
Peptidoglycan	PF01471	Putative peptidoglycan binding domain	1		2		1
	PF01476	LysM domain	9	1	4	1	5
Other	PF07554	Uncharacterized sugar-binding domain	1	4	1	1	2
	PF07564	Domain of unknown function (DUF1542)			1		3
	PF08428	Rib/alpha-like repeat	2	5			
	PF00041	Fibronectin type III domain				1	
	PF03217	Bacterial surface layer protein				11	
Cell wall anchor	PF00746	Gram positive anchor	5	6	5	4	13
	PF04650	YSIRK type signal peptide	1	7		5	17

[a] Using Pfam predictions at an e-value cutoff of 1E-3.

presumed to be lactobacilli, in comparison to gram-positive cocci, as well as gram-variable and gram-negative rods presumed to be anaerobic pathogens. Two scoring systems have been proposed, one by Nugent et al[20] called the Nugent system, and one by Hay et al,[21] which is a modification of a method first described by Speigel et al.[22] The Nugent system has been the most widely adopted to diagnose BV, however, more and more problems have been identified with it in recent times. The discovery of gram-positive rod-shaped *Atopobium vaginae* associated with BV[23] and its ability to produce lactic acid means that it could potentially sway the score toward normal when in fact the patient is not colonized with lactobacilli. It should be stated that health and susceptibility to disease are 2 different things; in the latter case, a woman may have a stable, nonlactobacilli microbiota which for her is asymptomatic and "healthy," but increases her risk of sexually transmitted infections.

These diagnostic systems do not take into account abundant aerobic organisms that account for over 10% of all aberrant samples.[13] In this case, the diagnosis should be AV not BV. Furthermore, concerns over the fields of view that are analyzed under the Gram stain methods and the difficulty in reproducibility between microscopes, especially for intermediate scoring, have been raised.[24]

In short, the most often used methods of diagnosing BV, the Amsel and Nugent scores, are often not in agreement with each other or accurate, nor do they correspond in any significant way with the self-reported symptoms.[25,26]

What are the Bacteria Doing?

To date, our understanding of what the bacteria do in the vagina is quite primitive and simplistic. The finding that most lactobacilli strains isolated from the vagina produce hydrogen peroxide (H_2O_2),[27] and this defense against pathogens[28] is flawed for several reasons. First, the ability of bacteria to produce this compound in a laboratory setting under aerobic conditions does not mean it can do this in a mostly anaerobic vaginal environment. Second, it is these H_2O_2 strains that are displaced when BV occurs,[1] suggesting the protection offered by this compound is ineffective even in allowing the lactobacilli to outcompete the pathogens. Third, the species most adapted to survival in the vagina, *L iners*, produces little or no H_2O_2; thus, from an ecological point of view, H_2O_2 does not appear to provide a major advantage to lactobacilli.

On the other hand, H_2O_2-producing species, such as *L crispatus* are clearly dominant in some healthy women.[1-3] Is this because the species or strains have certain properties different from less commonly found types like *L reuteri*? This has not been determined in full; but hypothesizing that adhesins could be important for persistent colonization, a comparison of

known genomes has not revealed any major properties found exclusively in *L crispatus* (Table 1).

A study of the genome of *L iners*, the *Lactobacillus* most universally found in the vagina,[29] including when patients have BV or are treated with antibiotics,[1] has revealed specialized adaptation mechanisms such as an iron–sulfur cluster assembly system, several unique σ factors to regulate gene transcription in the vagina, and a highly expressed homolog of a cholesterol-dependent cytolysin (CDC); all may contribute to persistence.[27] This species upregulates carbohydrate utilization genes during BV, as revealed by transcriptomic analysis (Macklaim et al, unpublished data), again illustrating its unique ecological and evolutionary adaptation to the vaginal environment. It remains to be determined whether all *L iners*, or just certain strains, use the CDC which is also significantly upregulated in BV (Macklaim et al, unpublished data) to extract nutrients from epithelial cells and thereby play a role in the onset or recovery from BV, or if this system simply takes advantage of an environment created by the dense BV pathogen biofilms on the epithelial cells. The difference in the function of *L iners* in a BV environment compared to a healthy vagina highlight our need to understand the conditional response and activity of the vaginal bacteria and not just their presence or abundance.

Preliminary metabolomic studies of vaginal samples have confirmed the presence of succinate and butyrate, products of anaerobic metabolism but not by *L iners* (McMillan et al, unpublished data; Ravel et al, unpublished data). This succinate peak also arises in patients who move from a normal to an *Atopobium*-donimated BV microbiota (Figure 1), again suggesting it is due to bacterial metabolism. Spear's group[30] have quantified the immunomodulatory short chain fatty acids and shown increased levels of acetate, propionate, and butyrate corresponding to increased anaerobes. Meta-transcriptomic analysis has revealed that the genes necessary for the production of these metabolites exist, and are active, in the organisms classically associated with BV (Macklaim et al, unpublished data).

The manner in which the host responds to the vaginal microbiota has been examined using human gene arrays in 3 studies. In premenopausal woman, the administration of *L rhamnosus* GR-1 to the vagina resulted in the upregulation of some antimicrobial genes.[31] On the contrary, studies of women with BV have shown impairment in cervicovaginal immune responses; and for postmenopausal women with vaginal dryness, there was also downregulation of epithelial integrity genes.[13,32,33] Such epithelial damage may be visible upon genital examination, and one such study found an increased prevalence in women with BV.[34]

Toll-like receptor 4 is stimulated, presumably by gram-negative anaerobes, in BV,[30] and this may play a role in bacterial binding and stimulation of interleukin (IL)-1b, but it is in AV that IL-1b and IL-6 are most significantly stimulated.[13] Higher levels of proinflammatory cytokines in cervicovaginal fluid may affect local HIV replication and increase the risk of acquisition or transmission of HIV[35] by attracting CD4 cells to the mucosa. Interestingly, glycogen accumulation in the epithelial cells is down during BV events, for unknown reasons, and as this is believed to be a key substrate for *Lactobacillus*, it may in part explain that genera's decline.[40] Supporting this hypothesis, studies of the vaginal microbiota of macaques have revealed lower glycogen levels than in humans and a correspondingly more "BV-like" microbiota with fewer lactobacilli.[36]

Another intriguing issue is the role that organisms such as *G vaginalis* have in the vagina, since they appear to be present in almost all women. It seems increasingly clear that there is more than 1 *G vaginalis*, and indeed 4 clones at least have been discovered.[37] The genus *Gardnerella* comprises a single species, *G vaginalis*, and a distinct clade within the *Bifidobacteraceae* family. A genomic comparison of 2 *G vaginalis* strains isolated from BV patients with almost identical 16S rDNA, a strain sharing only 98% 16S rDNA identity from a healthy woman, noted significant differences,[38] and supported the concept that detection of virulence expression should accompany detection of *G vaginalis* in the vagina, in order to know whether it is involved in infection. Whether the difference between being commensal and pathogenic related to adhesion to vaginal epithelial cells and cytotoxicity remains to be tested in vivo but is a possibility.[39]

What Do Probiotics Do?

As listed in Table 2, significant advances have been made in our understanding of probiotic lactobacilli and their potential to improve vaginal health. Some of the findings, such as upregulation of barrier function of the epithelium have been shown in intestinal epithelial models,[40] human amnion cells (Koscik et al, unpublished data), immortalized vaginal epithelial cells (http://www.ncbi.nlm.nih.gov/pubmed/22072832), and gene microarray studies (G. Reid, personal communication, April 20, 2012). While numerous products claim to be for vaginal health, 3 strains, *L rhamnosus* GR-1, *L reuteri* RC-14, and *L crispatus* CTV05 have been well studied in humans. The decision to select these strains is quite different: for the GR-1 and RC-14 combination, the rationale was that the organisms should be able to interfere with gram-positive and gram-negative pathogen growth and encourage restoration of the indigenous lactobacilli, while the CTV05 was selected because the species is commonly found in the vagina and produces large quantities of H_2O_2. Many properties of the GR-1/RC-14 strains have been investigated and recently summarized in a review[41]: these include their ability to modulate host defences,[31] produce antiadhesion and antivirulence factors,[42] and disrupt urogenital pathogen biofilms.[43] In human studies, the strains administered orally have been shown to reach the vagina, reduce pathogen ascension into the vagina, reduce recurrences of BV, prevent recurrences of UTI, enhance antimicrobial cure of BV and vulvovaginal candidosis, and shift the microbiota to be dominated by *Lactobacillus* sp.[41,44,45]

Phase 1 and 2 studies using an intravaginal drug containing *L crispatus* CTV05 have shown that when the organism persists for 28 days, number of pathogens *G vaginalis, A vaginae, Megasphaera* sp, *Leptotrichia/Sneathia* spp, and BVAB2 falls

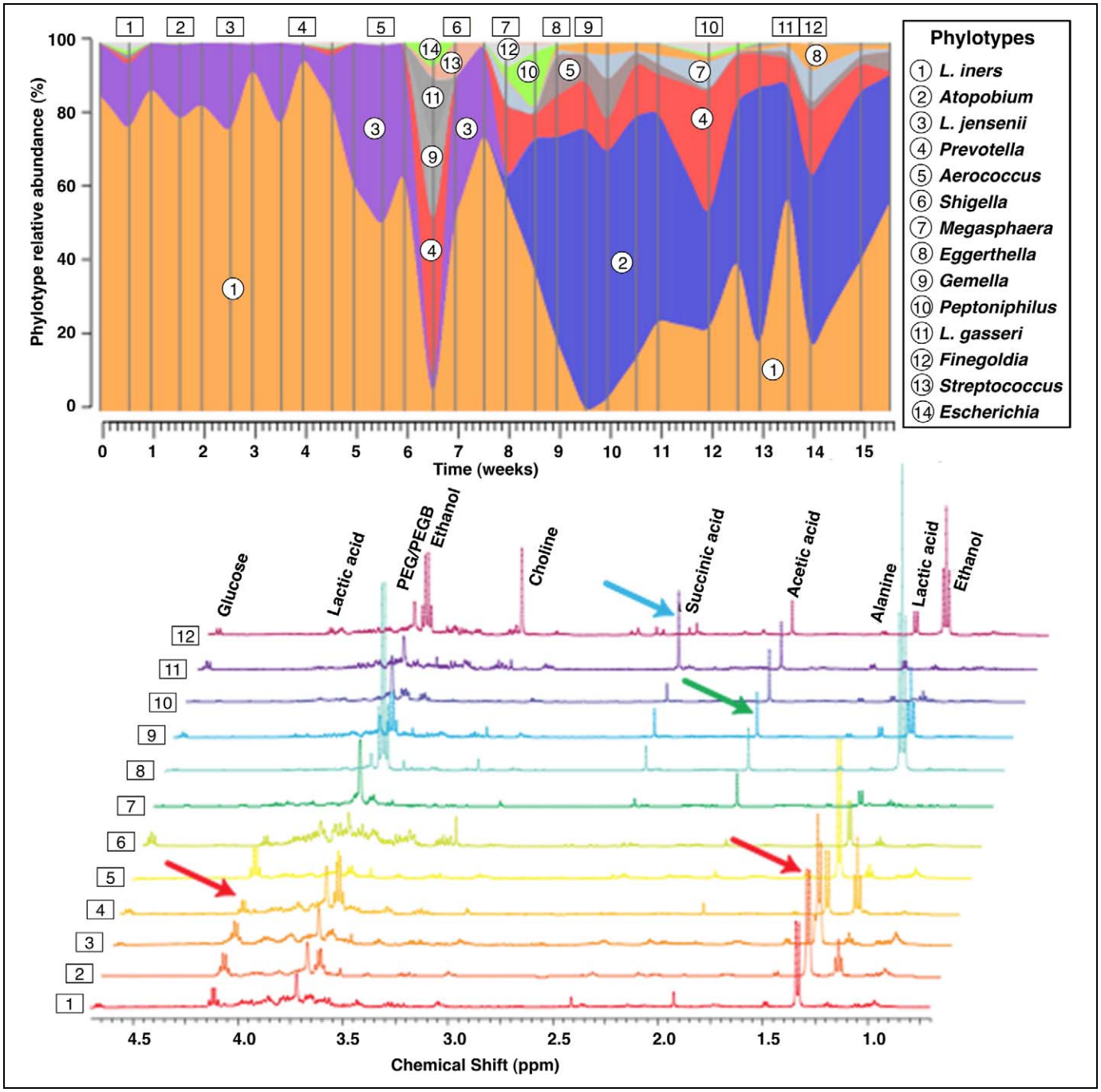

Figure 1. Courtesy of Dr. J. Ravel.[6]

significantly.[46] Another pilot study showed higher colonization was associated with reduced recurrences of UTI.[47] However, when the organism does not persist, likely because indigenous *L crispatus* are present, or antimicrobial therapy has failed to alter uropathogen counts, or there has been exposure to semen,[48] the effect on pathogens is marginal at best.

Another strain, *L acidophilus* KS400 has been used in conjunction with 0.03 mg estriol for local application to the vagina and shown to help restoration of lactobacilli after antibiotic treatment, as well as provide beneficial short-term effects on symptomatic BV.[49-51] In some of these effects, it is not clear how much is contributed by the addition of the small dosage of estriol.

In other studies, lactobacilli have been tested to deliver microbicides for the prevention of HIV spread through intercourse. Various concepts have been explored using *L reuteri* RC-14 expressing CD4D1D2-antibody-like fusion proteins,[52] and *L jensenii* 1153 expressing anti-HIV-1 chemokine RANTES, and mutated analogue C1C5 RANTES.[53] In addition, microbicides such as VivaGel (3% w/w SPL7013 in

Table 2. What Can Probiotics Do?

- Reduce symptoms and signs of disease
- Induce antimicrobial effects from the host
- Upregulate barrier function of the epithelium
- Modulate host immunity
- Disrupt pathogenic biofilms
- Enhance the effect of antimicrobial agents and possibly other drugs

Carbopol-based aqueous gel), a potent inhibitor of HSV-2 and HIV has been tested, but it induced vulvar and cervical erythema, cervical lesions, symptomatic BV, urinary frequency, and metrorrhagia.[54] As BV is a risk factor for HIV, rather than use microbicides that are toxic to the epithelium, the administration of lactobacilli and extended periods of maintenance of microbial homeostasis might also be worth pursuing to prevent HIV. Whether probiotics can also decrease the risk of HIV in women infected with human papilloma virus (HPV), itself a risk factor (new ref 55) remains to be seen, but one study suggests that HPV positive women treated with L. rhamnosus GR-1 and L. reuteri RC-14 have lower incidence of BV (new ref 56). The potential for long-term maintenance of a lactobacilli-dominated vagina is being explored with *L crispatus* CTV05.[55]

The Road Map

The plight of women with urogenital disease is massive and major efforts are needed to address this burden. The following is a road map that identifies 3 areas in need of urgent attention: (1) improvement in education of women and health care practitioners on the vaginal microbiota; (2) targeted funding for research to improve diagnosis and test new therapies; and (3) ensure that new approaches are accessible in developing countries and empowering to women, as well as being acceptable and appropriate for different populations.

Education

Educational programs accessible to as many females as possible (by necessity in different languages) are needed to explain urogenital diseases, the natural role that microbes play in maintaining health, and hygienic practices that can help or hinder maintenance of microbial homeostasis. This should differentiate pain, discomfort, and signs such as reddening and discharge from UTI versus vaginal infections by viruses, yeast, and bacteria. Knowledge is the first stage of empowerment, and for many women simple anatomy and issues that arise through the menstrual cycle have never been adequately explained. Such education must avoid any perception of guilt for such conditions.

For physicians, there is an urgent need to update their knowledge of the vaginal microbiome, the different profiles associated with health and with disease, the many limitations of current diagnostic methodologies, the narrow efficacy and poor specificity of antimicrobial therapies, the expectation of recurrences and the burden that lower urogenital symptoms place on well-being and quality of life.[56] In addition, there needs to be a consensus drawn on what tests can be done in different settings (private practice, clinics, hospitals, rural areas), what information can be gained from each, and how to interpret the findings. When symptoms are reported, on-site wet mount microscopy is arguably the most revealing in diagnosing AV, BV, or yeast infection and a normal condition,[14] and, contrary to common perception, the learning curve of fresh wet mount microscopy is relatively short, provided proper training is administered (Donders et al, submitted for publication). Requesting cultures is unlikely to be revealing except for detection of *Trichomonas* and the confirmation of *Candida* in patients suspected of recurrent vulvovaginal candidosis but with negative microscopy. In all other cases, just the isolation of yeast does not necessarily equate to infection. Hopefully, with increased education, physicians seeing women with BV and AV will be able to consider the use of clinically documented probiotics as a therapeutic adjunct.

New Diagnostics and Therapies

Now that there is a better understanding of the types of bacteria present in the vagina, the next steps are to identify the other classes of organisms (viruses, fungi, and protozoa), more fully the environmental metabolic outputs (the metabolome), as well as how the organisms and host function over time. Such research studies are critical if novel approaches to therapy are to be created, and funding for such fundamental science will only be forthcoming if government and philanthropic agencies begin to appreciate the seriousness of urogenital disease and its complications. Such studies can identify longitudinal microbial profiles that correlate with highest risk of disease and its complications (eg, preterm labor, cervical cancer) and how the host can sometimes self-modulate these profiles back to normal. By identifying subgroups of women, it becomes possible to improve responder rates and stratify participants in longitudinal studies.

The potential benefit of probiotic and prebiotic intervention is good, but studies are required to understand how they work and in whom. It may be that different probiotics are required for different microbiota profiles that are clinically deemed to be healthy. Also, in some cases, addition of small amounts of substrate-enhancing products like estriol may improve the effect of probiotic action on vaginal health.

In all clinical samples, the better defined the patient and the more samples that can be obtained, the better will be the data that are acquired. Standardized protocols for reading diagnostic methods (eg, differentiating *Atopobium* from *Lactobacillus* gram-positive rods; numbers of fields of view evaluated, or number of epithelial cells examined) would be helpful across centers, with samples appropriately stored for later analysis, for example on the metabolome.

Empowerment

Much can be learned from resource-disadvantaged settings, for example in Africa. There, interventions must be thoroughly

explained prior to their introduction and use and an appreciation and respect given to local community and their cultural ways. Thus, for example, women in Western Europe, United States, and Canada might be fully able to collect their own vaginal swabs and deliver it to the laboratory, while women in rural Africa may not be familiar with their urogenital area and be uncomfortable with even a doctor taking a vaginal sample. Some women are very interested in self-testing for vaginal microbiota abnormalities, and positive toward understanding the risks of abnormal test results and accepting treatment to correct this.[57] Such differences between people are not confined to African countries and need to be considered in all cases. Sensitivity to cost is also required, and arguably this is becoming more and more relevant in North America where companies charge disproportionately higher fees for drugs and diagnostics, yet more of the population is facing economic challenges.

The ideal interventions are those which any woman can use with ease and comfort while maintaining privacy. These should allow easy interpretation of findings along with an explanation of what any given result means. Thus, if a diagnostic kit accurately identified a pregnant woman at risk of preterm delivery because of microbiota and host changes, what does the recipient of this information need to do next? It might be obvious to visit a doctor, but if they are far away, or unavailable, or the person has no money to go there, what are the consequences for the person and her baby? Are there things she can do to reduce the risk of miscarriage? An advantage of a probiotic yogurt produced locally at a very low cost, and for example shown to improve vaginal microbiome and immune status, is that a woman can gain easy access to it and is thereby empowered. Too often, expensive products are tested on poverty-stricken participants, then after the trial the participants cannot afford to buy them or the products are not available. In the future, neglecting the women who need treatment the most must be regarded as being unacceptable. Likewise, not seeking their input into such interventions makes the likelihood of implementation diminished. The successful integration of cervical cancer prevention in HIV/AIDS treatment and care programs in Zambia[58] demonstrates that novel therapies can be transferred to the betterment of women around the world.

Having made these points, it is clear that money drives product development, and industrial partnerships will be needed to create novel and better diagnostic systems and drugs, as well as medical foods and natural health products that are effective. For now, drugs such as metronidazole continue to be used in most cases to treat symptomatic BV, even if it was never created to treat the organisms causing the disease.

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