Review Article

NEW FINDINGS ABOUT VAGINAL BACTERIAL FLORA

Iara Moreno Linhares[1*], Paulo Cesar Giraldo[2], Edmund Chada Baracat[3]

Study conducted at Disciplina de Ginecologia da Faculdade de Medicina da Universidade de São Paulo – FMUSP, São Paulo, SP, Brazil

ABSTRACT

The aim of this review is to update knowledge about the vaginal ecosystem, emphasizing non-cultivation methods for bacterial identification (gene amplification), the several Lactobacillus species that comprise normal vaginal flora and influence of host genetics on bacterial interactions with local innate and acquired immune defenses. A Medline (Pubmed) search from 1997-2009 for relevant articles was performed and the most informative articles were selected. The use of non-cultivable techniques (genes amplification techniques) have enabled a better knowledge about the composition of the vaginal ecosystem. In most women in the reproductive age there is a predominance of one or more species of *Lactobacillus: L. crispatus, L. inners and L gasseri*. However, in other apparently healthy women there is a deficiency or complete absence of Lactobacilli, which are substituted by other lactic acid-producing bacteria: Atobium, Megasphaera and/or Leptotrichia species. The infectivity and/or proliferation of pathogenic bacteria in the vagina is suppressed by lactic acid production, by products of endogenous bacteria and by activation of local innate and acquired immunity. Vaginal epithelial cells produce several compounds with antimicrobial activity. These cells have Toll-like receptors on their membrane that recognize molecular patterns associated with pathogens. Recognition leads to production of pro-inflammatory cytokines and stimulation of antigen-specific immunity. The production of IgG and IgA antibodies is also triggered in the endocervix and vagina in response to infection. Vaginal flora composition and the immune mechanisms constitute important defense mechanisms. Criteria of normal and abnormal flora have to be reviewed and genetic polymorphism can explain variations in flora composition. This new knowledge should be included in the clinical practice of gynecologists and obstetricians to improve patients care.

***Correspondence:**
Av. Dr. Enéas de Carvalho Aguiar, 255 - 10º andar - sala 10.166 - Disciplina de Ginecologia
São Paulo – SP, Brazil
CEP: 05403-900

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Introduction

Anatomically, the female genital tract is constituted by a succession of cavities (Fallopian tubes, uterine cavity, endocervix, vagina) that communicate with the exterior through the vulvar cleft. This structure allows the exteriorization of the menstrual flow and the passage of the fetus at delivery; on the other hand, it allows the sexual act and also the entrance of pathogenic microorganisms potentially harmful to the reproduction process. Vaginal microflora undoubtedly presents one of the most important defense mechanisms for the reproductive function, maintaining the environment healthy and preventing the proliferation of microorganisms stranger to the vagina.

The first studies on the flora were performed by Doderlein, who has identified *Lactobacilli* as constituents of the healthy flora. Since then the diverse components of the vaginal ecosystem have been observed on the microscope and, afterwards, identified through culture-specific techniques.[1] However, more recently, identification techniques for independent bacteria in the means of culture have revolutionized the study of microorganisms. The use of amplification, cloning techniques and subsequent analysis of sequences of bacterial genes (genes that codify for bacterial rRNA 16 S) in samples of vaginal fluid have allowed the identification of the majority of common species of *Lactobacilli* and other microorganisms. Thus, these techniques have demonstrated that *Lactobacilli sp* do not always correspond to the dominant species in the vagina of healthy women. Besides that, vaginal environment inhabitants until then unknown have been identified.[2,4]

1. Pós-doutorado - Coordenadora do Setor de Imunologia Genética e Infecções do Trato Reprodutivo da Disciplina de Ginecologia da Faculdade de Medicina da Universidade de São Paulo – FMUSP, São Paulo, SP
2. Livre-docente - Professor Associado do Departamento de Tocoginecologia da Universidade Estadual de Campinas – UNICAMP, Campinas, SP
3. Livre-docente - Professor Titular da Disciplina de Ginecologia do Departamento de Obstetrícia e Ginecologia da Faculdade de Medicina da Universidade de São Paulo – FMUSP, São Paulo, SP

Lactic acid-producing bacteria

Although vaginal bacterial species of healthy women during menacme have been initially identified as *Lactobacillus acidophilus*,this knowledge represents a simplification, since in women whose flora is dominated by *Lactobacillus,* the more frequently detected species through genic amplification are *L crispatus* and *L. inners*[2,4] or *L. crispatus and L. gasseri*[3]. Other species as *L. jensinii , L. gallinarum* and *L. vaginalis* have also been identified in some women. A study on vaginal flora accomplished in three continents using methods of bacterial genes analysis showed that dominant species were the same in each region: *L. crispatus, L. gasseri* and *L. jensinii*[5]. Besides that, the same study observed that, in some women, the normal ecosystem was maintained in the absence of *Lactobacillus;* in one woman *Atopobium vaginae* was identified as the dominant microorganism in the flora and, in two other women, the bacteria *Atopobium, Megasphaera and Leptotrichia* were all lactic acid producers similarly to *Lactobacillus.* [2,6] Therefore, the vagina's acid environment, known as an important defense mechanism against the proliferation of pathogens, may be kept by other bacteria, not only by *Lactobacillus.* It is possible that when the *Lactobacilli* are not capable of predominating in the vaginal environment due to any reason, other lactic acid-producing bacteria start occupying their place. It must be highlighted that the *Megaesphaera* and *Laptotrichia* bacteria are also capable of producing unpleasant smelling metabolites. Therefore, the detection of vaginal odor in women that do not have a vaginal flora dominated by *Lactobacillus* is not conclusive to perform the diagnosis of pathological entities as, for instance, bacterial vaginosis, especially in asymptomatic women. Similarly, the variable morphology of *Atopobium*, varying from elliptic cocci to organisms with the shape of curved bacillus, occurring in pairs or chains,[3] makes its detection by bacterioscopy through the Gram method or by the fresh examination problematic, because this microorganism may be easily confused with other bacteria associated to the picture of bacterial vaginosis.

Biofilms

One of the difficulties found for a broader characterization of the vaginal microbial flora is the presence of biofilms. Biofilms are formed by colonies of microorganisms that adhere among themselves and cover a solid surface. Biofilms have already been identified in the surface of vaginal cells, more known in women with bacterial vaginosis, where species of *Gardnerella vaginalis* and *Atopobium* predominate.[7] However, an interesting aspect to be considered is the study of the biofilms in asymptomatic women, which were not yet characterized.

Race/Ethnicity

The possible differences in the composition of the 'normal' vaginal flora still need to be more studied. The occurrence of species of *Lactobacilli* producers of hydrogen peroxide, which possess a defense activity against pathogens, seems to be less frequent in African-American women.[8] Studies suggest that, for bacterial vaginosis-free women, the vaginal pH is higher in African-American women;[9] they suggest also that such differences are valid only for women whose vaginal microflora is not dominated by *Lactobacillus.*[10,11] The higher prevalence of bacterial vaginosis detected by the Gram method in African-American women may merely reflect the presence of bacteria other than *Lactobacillus*, as predominant in the African-American population, which does not mean that these women are bacterial vaginosis carriers.

Fluctuations in vaginal flora

The composition of the vaginal flora is not constant, suffering variations in response to exogenous and endogenous factors.[12,15] These factors include the different phases of the menstrual cycle, gestation, use of contraceptives, frequency of sexual intercourse, use of showers or deodorant products, use of antibiotics or other medications with immune-suppressive properties. Alterations occurring in the vaginal environment may increase or decrease the selective advantages for specific microorganisms. For example, studies have related the loss of *Lactobacillus* to sexual intercourse or to the use of antibiotics.[14] Nevertheless, another study has demonstrated that the sexual act without the use of condom had no effects upon the *Lactobacillus,* but increased the level of *Escherichia coli* and facultative gram- negative bacilli.[15] During the menstrual cycle, hormonal variations interfere in the substrate of different microorganisms; these variations, as well as menstrual blood, lead to changes in vaginal pH. Yet the levels of *Lactobacillus* remain constant throughout the cycle, the non-*Lactobacillus* bacteria increase during the proliferative phase and the concentrations of *Candida albicans* become higher in the pre-menstrual period.[13]

Antibiotics may alter the vaginal ecology, therefore the treatment of asymptomatic women, although with a fresh examination showing the absence of *Lactobacillus,* is very questionable. To induce a perturbation in the endogenous microflora based solely on the microscopic findings may trigger the selective proliferation of microorganisms that were being inhibited and may be damaging to the vagina's health.

Candida albicans is tolerant to the acid environment, found in approximately 10% to 20% of women in reproductive age. The concentration of the microorganism is low, so the woman carrier is asymptomatic. Nevertheless, events leading to a state of local immune-suppression, such as sexual intercourse or local induction of allergic response, create adequate conditions for the proliferation of the microorganism and also facilitate the transformation into the shape of hyphas, more invasive.[16] These situations result in the emergence of symptomatic vaginitis.

The production of lactic acid may be essential for the maintenance of a healthy ecosystem, regardless of the bacterial species that may be present in the vagina. Resulting acid pH prevents the excessive proliferation of potentially pathogenic microorganisms. It is important yet to remember that the *Lactobacillus* dominance is beneficial for the host, since some species produce hydrogen peroxyde and bacteriocins, factors that hinder the proliferation of other microorganisms.

Genetic polymorphisms

Genetic polymorphisms are small changes in the DNA sequence of a gene, occurring in healthy individuals. Generally they involve both an alteration in a single pair of bases or a variation in the length of a repetitive DNA sequence. The polymorphism may occur in the region promoting the gene, that

is, in the part of the gene that is not transcribed in protein, but influences the transcription rate. Other possibility is the occurrence, in the gene's codifying region, the region influencing the final composition of the protein, determining the substitution of one aminoacid by another. Such changes may result in alterations in the conformation of the protein's composition and activity.

Studies have demonstrated that the individual genetic capacity of producing low or high levels of anti or pro-microbial factors represents an important variable, which influences the composition of the vaginal microbial flora and the response to pathogens. Polymorphism in genes such as the interleukin 1 receptor antagonist or the 'Toll-like receptor 4', a cell surface receptor that acts in the innate recognition of gram-negative bacteria, may influence the quantitative composition of bacteria in the vagina.[17,18] The frequencies of different genetic polymorphisms vary between the different ethnical-racial groups; this fact may be associated with the differences in the composition of the ecosystem between different populations.

Vaginal immunity

In addition to the protective effects of the endogenous vaginal flora, the protection against potentially pathogenic microorganisms is done also by the local components of innate immunity and acquired immunity.

The innate immune system is the most primitive branch of the immune system, having been conserved throughout evolution. One of its characteristics is recognizing molecular patterns associated with the pathogens (PAMPs) in the invading microbes, instead of recognizing specific antigens. Factors of innate immunity acting in the vagina are represented by soluble factors (such as mannose-binding lecithin [MBL], components of the complement, defensines, secretory leukocytes protease inhibitor [SLPI], nitric oxide), components associated to the membrane ('*Toll-like receptors*') and phagocytic cells. The recognition of a PAMP by a component of the innate immunity triggers a sequence of events leading to the release of pro-inflammatory cytokines and, consequently, the activation of the acquired immune system, which is, the activation of lymphocytes T and B. Once these lymphocytes are activated, they initiate the immunity mediated by cells and humoral immunity. It is important to highlight that, while the activation of the innate immune system occurs immediately after the recognition of the pathogen, several days are necessary for the acquired immunity to become functional.

The epithelial cells layer of the vagina constitutes the initial contact point between microorganisms and the host's genital tract. These epithelial cells possess '*Toll-like receptors*' (TLR) in their surface and, therefore, are important components of innate vaginal immunity.[19] Eleven TLRs have been identified already, each with a different specificity.[20] TLR1 and TLR2 complexes recognize lipoproteins and peptideoglycan present in the surface of gram-positive bacteria. TLR3 is specific for the double DNA chain, which is an intermediate in the replication cycle of many viruses. TLR4 recognizes the liposacaride component of the gram-negative bacteria wall. TLR5 reacts with flagellins, components of the bacterial flagella. TLR9 has the capacity of distinguishing DNA sequences containing the CpG dinucleotide in the non-methylated state (in humans the DNA sequence is highly methylated, but the TLR9 reacts only with the CpG of bacteria, non-methylated, being, then, specific for them).

Vaginal cells also release molecules with potent non-specific antimicrobial activity. A class of these molecules, known as defensines, include positively-charged peptides, that rapidly bind negatively-charged bacterial surfaces. This binding results in the disruption of the microorganism membrane and in cellular lysis.[21] Human defensines HBD-1 and HBD-2 are produced by vaginal epithelial cells. In women with infections, the production of HBD-2, but not that of HBD-1, is stimulated by estrogens and inhibited by progesterone.[22] This suggests that the use of oral contraceptives may decrease the release of HBD-2, increasing, thus, the susceptibility to infections. Another class of molecules is represented by the SLPI, which possesses the capacity of inhibiting enzymes that break proteins (proteases), destroying gram-positive and gram-negative bacteria and blocking the action of the human immunodeficiency virus.[23] The production of SLPI also occurs in the uterine tube.[24] Vaginal levels of this protein are decreased in women with bacterial vaginosis,[25] suggesting that the SLPI, as a component of the innate immune system, plays an important role in the vaginal homeostasis maintenance.

Mannose-binding lecithin (MBL) is an antimicrobial protein present in the vaginal circulation and secretion. It is synthesized primarily by the liver, the vaginal production being still uncertain. The MBL recognizes and binds mannose residues, N-acetylglucosamine and fucosis present in the surface of microorganisms. This binding induces the activation of the complement system and deposition of its components in the microbial membrane. This fact induces the direct lysis of sensitive bacteria or their opsonization.[26] Studies have demonstrated that women with MBL deficiency due to a polymorphism in the gene's codifying region regulating the production of this protein are more susceptible to recurrent infections by *Candida albicans.*[27]

Heat shock proteins are among the more highly conserved proteins in the evolution of living beings. They are fundamental for the conservation of life in each organism known and they help the cell to survive in various environmental conditions, such as the exposition to high temperatures, chemical poisons, inflammation or aggression by microbial pathogens. The heat shock protein HSP-70kDa (hsp70) was recently recognized as one of the antimicrobial proteins present in the vagina.[28,29] The hsp70 synthesis is highly stimulated in response to inflammation and infection, being possibly located inside and/or outside the cell. Intracellular hsp70 binds other proteins (under adverse conditions) and avoids their degradation.[30] Extracellular hsp70, on its turn, binds 'toll-like receptors,' thus stimulating the immune response to pathogens.[31] Studies suggest that hsp70 produced in response to abnormal vaginal microflora induces the release of nitric acid in the vagina.[32] The nitric oxide has an antimicrobial activity against a wide variety of microorganisms.[33]

Besides SLPI,[23] other components of the innate immune system protecting against infections by retrovirus, particularly HIV, have been characterized in the female genital tract. CCL20/MIP3 alpha chemokine is produced by uterin or tubarian cells; in vitro studies with cells cultures show an increase in the production of this protein after stimulation with double chain of synthetic DNA homologue to the viral RNA chain.[34]

Antibodies with the ability of recognizing and binding specific microbial antigens are found in the vagina by transudation of

the systemic circulation; after the binding comes the microbial death, by complement-dependent mechanism, or by opsonization. Moreover, a component of the mucosas' immune system is found in the reproductive tract. Thus, antibodies-producing B-lymphocytes are present in the endocervix and also in the vagina, locally producing both classes of antibodies, IgC and IgA.[35] The local antibodies elaboration represents a rapid mechanism for fighting pathogenic microorganisms, without the need to wait for the beginning of systemic immune response. Antibodies formed locally and present in the vagina probably differ from the systemic ones; moreover, it is possible to identify antibodies in the cervical-vaginal secretion that are not detectable in peripheral blood.

Conclusions

1) Vaginal bacterial flora, associated with the presence of various components of innate and acquired immunity, constitute an important defense mechanism to avoid invasion/proliferation of microbial pathogens in locals exposed to external environment.

2) Advances in the non-cultivable methods of genes amplification for the identification of bacteria have allowed attempts to characterize a more definitive picture of the vaginal ecosystem composition. Until the beginning of these studies, vaginal flora was divided into two categories: 'normal flora,' dominated by *Lactobacillus* and 'abnormal flora,' with the dominance of other bacterial species. However, new microorganisms identification techniques have allowed the understanding that, in some women, a vaginal flora not dominated by *Lactobacillus* may be 'normal' and not necessarily pathogenic.

3) Advances in the study of genetic polymorphisms explain variations in the individual functioning in the production, concentration or effectiveness of the innate immune system's components. Then, if the characteristics of the woman studied are not taken into consideration, observational studies and clinical tests performed considering a specific vaginal flora will be of very limited usefulness, especially those based on the smears with coloring by the Gram method or other morphological characteristics.

4) The sequence of events culminating with the development of bacterial vaginosis, an entity characterized by deep alteration in the physiological vaginal flora, still need to be determined. In face of new bacterial genetic studies, it is questioned if all the women with 'asymptomatic bacterial vaginosis' diagnosis, performed based solely on the microorganisms' morphologic characteristics, are really carriers of a flora disorder or if they simply have a normal vaginal ecosystem that is not dominated by *Lactobacillus.* This fact must be carefully determined based on individual characteristics, instead of merely submitting these women to unnecessary treatments, probably damaging to their vaginal flora's particular balance.

5) It is fundamental that gynecologists and obstetricians broaden their knowledge on vaginal microbiota and recent immunology studies, bringing their results into their practice. Besides that, it is important for more advanced laboratory techniques to surpass the limits of the research laboratories and become quickly available in the clinical practice, thus becoming able to be incorporated in the improvement of care. The use of new knowledge and more advanced diagnostic possibilities will certainly contribute to the improvement of reproductive health practices, avoiding unnecessary treatments and improving the women's quality of life.

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