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Review Article

Vaginal Dysbiosis

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Abstract

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Key Words: dentistry; techniques; computer

Introduction

Dysbiosis is a condition in which the balance of the microflora is disrupted: there are fewer beneficial bacteria and more opportunistic and pathogenic bacteria.

Bacterial vaginosis (BV) is the most common non-inflammatory disease of the vagina, affecting 30% of women worldwide. BV is associated with an increased risk of a wide range of gynecological and obstetric complications, including premature birth, spontaneous abortion, early pregnancy loss in IVF, and infection and transmission of HIV/STDs. Although up to half of women with bacterial vaginosis do not experience any symptoms (4, 54), those who do experience symptoms often report significant discomfort and impact on their quality of life and relationships (11).

Etiology and pathogenesis

Normally, the vaginal microbiota of a healthy woman of reproductive age is 95-98% represented by lactobacilli (Lactobacillus spp.), their main functions:

-Production of lactic acid from glycogen, creating a low pH (3.8-4.5), which inhibits the growth of opportunistic pathogens;

-Synthesis of hydrogen peroxide and bacteriocins, which have direct antimicrobial activity;

-Competition for adhesion receptors and nutrients.

Current evidence suggests that bacterial vaginosis is a polymicrobial syndrome characterized by a shift in the vaginal microbiota from "optimal" to "suboptimal" (24, 29, 34, 67). This suboptimal microbiological condition is characterized by a decrease in the number of protective lactobacilli and an increase in the diversity of bacteria, as well as facultative and strict anaerobes, including Gardnerella spp., Atopobium vaginae, Prevotella spp., and others, which are known as bacteria associated with bacterial vaginosis (BV) (29, 82). Although the exact causative agent/agents of bacterial vaginosis have not yet been established, a recent conceptual model suggests that virulent strains of Gardnerella, as well as Prevotella bivia and A. vaginae, play a central role (74). Also to the alleged causes of the development of the disease include: antibiotic therapy, which destroys both pathogenic and normal bacteria; hormonal changes, menopause, pregnancy or the use of hormonal contraceptives can change the level of estrogens and, accordingly, the composition of the microflora; improper intimate hygiene, the use of aggressive detergents or frequent washing can disrupt the natural balance; a decrease in local immunity can contribute to the development of dysbiosis, as the body is less effective in dealing with pathogens.

Risk factors for the development of BV:

-frequent changes of sexual partners;

-douching, which destroys the natural microflora;

-prolonged and uncontrolled antibiotic therapy;

-use of intrauterine devices;

-immunodeficiency conditions.

Clinical picture

Vaginal dysbacteriosis may manifest itself by itching, burning, change in smell ("fishy") and color of vaginal discharge: abundant, homogeneous, liquid discharge of grayish-white color, increasing after sexual intercourse or menstruation.

Diagnosis

The diagnosis of bacterial vaginosis is usually made based on the Amsel criteria or the Nugent scale (Redelinghuys et al., 2020). The Amsel criteria evaluate the clinical symptoms associated with bacterial vaginosis, and a positive diagnosis is made if three of the four criteria are present (Amsel et

al., 1983). These criteria include: characteristic discharge, a vaginal pH greater than 4.5, a positive amine test (the appearance of an odor when a 10% KOH solution is added to the discharge), and the detection of vaginal epithelial cells heavily covered with bacteria under microscopy. When using the Nugent scale, a microscopic analysis of the vaginal swab is performed, and the number of morphotypes in the sample is counted and classified using a semi-quantitative scale. A score of > 7 indicates a positive case of BV (Nugent et al., 1991).

Additionally, real-time PCR can be used to quantify the entire spectrum of the vaginal microbiota. Molecular tests can be performed to detect G. vaginalis, A. vaginale, and their ratios, enhancing the accuracy of diagnosis. Five highly sensitive and specific multiplex PCR tests are available (BD Max™ Vaginal Panel (36), Hologic Aptima® BV (48), LabCorp NuSwab® VG (14), Quest Diagnostics™ SureSwab® Bacterial Vaginosis, and Medical Diagnostics Laboratory (MDL) OneSwab® (45)). These tests include various combinations of Lactobacillus spp. in addition to G. vaginalis, A. vaginae, BVAB2, and Megasphaera-1 and -2.

Molecular diagnostic methods (including direct probe assays, NAAT, 16S rRNA sequencing, shotgun metagenomic sequencing, and fluorescence in situ hybridization) for BV have advantages over traditional diagnostic methods because they do not require the use of microscopy or other on-site procedures, which reduces the burden on busy clinicians. They are also objective, as they are based on the detection of specific bacterial nucleic acids and are capable of detecting BVABs; many of them can be performed on both self-collected vaginal samples and samples collected by a doctor (16, 72). In addition, some BV NAATs are capable of detecting other microorganisms in addition to BVAB (e.g., Candida spp. and T. vaginalis) (37, 60). However, one limitation of these new methods is their higher cost compared to traditional BV diagnostic methods. In addition, some of these tests are either not yet commercially available or are not yet the preferred method for diagnosing BV in national guidelines (107) (e.g., 16S rRNA gene sequencing, shotgun metagenomic sequencing, and fluorescence in situ hybridization) and are currently used primarily for research purposes.

Direct probe assays introduce a DNA probe into a sample of vaginal fluid. The probe then binds to specific sequences of a particular bacterium in the sample and can detect the presence of different bacteria in a single sample (16). One example of a widely used direct probe assay used in BV diagnosis is the Affirm VP III assay (Becton Dickinson, Sparks, MD). This is a moderately complex test with a DNA probe that detects high concentrations of G. vaginalis nucleic acids (> 5 × 10 5 CFU G. vaginalis / ml) in vaginal fluid, with results available within 30 minutes to 1 hour. The sensitivity is 90% and the specificity is 97% compared to the detection of key cells on vaginal wet mounts, while the sensitivity is 94% and the specificity is 81% compared to the Nugent scale (16). This test is most useful for women with symptoms in combination with a vaginal pH measurement and the presence of an amine odor (sensitivity increases to 97%) (16). This test can also be used to detect Candidaspp. as well as T. vaginalis; however, it is not FDA-approved for the diagnosis of T. vaginalis in men. However, its use is limited because it only detects G. vaginalis for the diagnosis of BV and does not detect other BVABs. This is problematic because G. vaginalis is found in sexually active women with normal vaginal microbiota (8), and colonization with G. vaginalis does not always cause BV (44).

NAATs, such as PCR, can detect a single microorganism in a vaginal sample (16); these tests are more sensitive than direct probe assays. Quantitative PCR (qPCR) is used to quantify the number of copies of a given DNA template. Quantitative assessment of bacterial species in the vaginal microbiota using qPCR is a popular tool for identifying and measuring specific vaginal microorganisms for research purposes (30, 111). This method is accurate, but it requires a lot of time and the construction of a standard curve for each microorganism of interest (99). Research is still ongoing to determine specific thresholds for these microorganisms (111) and the concentrations at which key BVAB contribute to BV pathogenesis. qPCR has limitations, including the fact that probes must be developed for each microorganism of interest that target the amplicon within that microorganism but do not cross-react with target DNA sequences in other microorganisms. When developing qPCR primers, it is also necessary to rely on existing sequence databases, which may be incomplete. In this regard, the development of a primer specific to the microorganism under study is the most expensive process. An additional limitation is the cost, which may increase as the number of microorganisms under study increases. In addition to multiple target organisms, other sequencing methods such as 16S rRNA gene sequencing and metagenomic shotgun sequencing can be considered, as the cost of sequencing a single sample does not depend on the number of microorganisms under study. Nevertheless, qPCR is still a valuable tool for better understanding the microenvironment of the vaginal tract and developing commercial NAAT tests for the diagnosis of bacterial vaginosis.

In this direction, Fredricks et al. developed a panel of taxon-specific 16S rRNA gene PCR assays for detecting 17 species of vaginal bacteria (31).

Using vaginal samples from 81 women with BV and 183 women without BV, they estimated the prevalence of each of these vaginal bacteria. Women with BV had an average of 11.1 species (range: 5 to 16). In contrast, women without BV had an average of 3.6 species (range: 0 to 14). Detection of either BVAB2 or Megasphaera type 1 had a sensitivity of 95.9% and a specificity of 93.7% compared to the Nugent score (31). After this study and given the polymicrobial nature of BV, quantitative multiplex PCR assays became the focus of development for commercial BV diagnostics (16). Multiplex PCR uses unique sets of primers and probes that bind to sections of the 16S rRNA gene, allowing for quick and easy molecular diagnosis of bacterial vaginosis (BV) using proprietary algorithms for each analysis (16). Different BVs have different positive predictive values for BV diagnosis when used individually. However, the combined detection of multiple BVs can improve the test performance (16).

As of January 2023, there were six multiplex NAATs available in the US for BV diagnosis in cisgender women. These tests include the BD MAX vaginal panel (Becton Dickinson, Sparks, MD) (37), Aptima BV (Hologic, Marlborough, MA) (87), GeneXpert Xpress (MVP) multiplex vaginal panel (Cepheid, Sunnyvale, CA) (60), NuSwab VG (LabCorp, Burlington, NC) (13), the OneSwab BV PCR panel with Lactobacillus profiling using the KPCR method (Medical Diagnostic Laboratory, Hamilton, NJ) (46) and SureSwab BV (Quest Diagnostics, Secaucus, NJ). Three of them are FDA-approved for use in women with symptoms (BD MAX vaginal panel, Aptima BV, and GeneXpert Xpress MVP), while the others are laboratory-developed tests that must undergo internal validation before use. All FDA-approved tests for BV diagnosis have excellent sensitivity, which is a prerequisite for approval. They have not been shown to differ significantly in accuracy; however, there are several studies that provide direct comparisons of these tests (60). Specific vaginal bacterial targets included in each test, relative cost, sample types, and time to result. It is important to note that the vaginal bacterial targets included in the tests vary due to the etiology of BV, which remains poorly understood. These tests can be performed on both doctor-collected vaginal samples and self-collected samples, with results available within 60 minutes to 24+ hours, depending on the molecular diagnostic platform used. The use of these tests eliminates the need for microscopy, reading experience, and equipment maintenance, which are requirements when Amsel criteria or Gram-stained vaginal criteria (Nugent scale or Izod-Hay criteria) are used to diagnose BV. In addition, it has been shown that the use of the NAAT test for BV has a higher sensitivity and specificity for BV (≥96.2% and ≥92.4%, respectively) than physician-diagnosed BV (83.4% and 85.5%, respectively) and in-clinic assessments (75.9% and 94.4% for Amel criteria, respectively) in one study (87). However, these tests are more expensive than traditional methods of BV diagnosis (71), have not been studied among transgender groups, and are only recommended for use in symptomatic cisgender women (107), do not determine the severity of BV

symptoms, and do not differentiate between persistence, relapse, or reinfection in women with recurrent BV. Additionally, it is challenging to define a gold standard for comparing the effectiveness of new NAATs for BV, although the Nugent scale is often used for evaluation. This limitation does not allow for an accurate determination of the clinical sensitivity and specificity of commercially available NAATs, and it should be considered when evaluating these new tests for use in laboratories.

To overcome the limitations of real-time PCR (qPCR), researchers have been looking for universal genes that exist in all microorganisms of interest. The 16S rRNA gene was chosen for the analysis of bacterial communities (106). Universal primers have been designed to ensure the amplification of a specific part of this gene, the amplicon, to apply PCR to the entire pool of DNA extracted from the bacterial community (9). In practice, these primers achieve more than 95% amplification, missing only a small fraction of the microorganisms in the community, depending on the chosen primer (100). To achieve a high level of universality, primers are often degenerate, including a mixture of closely related sequences (86). After amplification of the amplicon in question, which is also ligated with sequencing primers, the resulting DNA can be subjected to sequencing analysis (50). Initially, only the Sanger sequencing method was available, allowing researchers to assess the full range of microorganisms present in the community, which could not be done using individual PCR assays. With the advent of less expensive high-throughput sequencing technologies in the early 2000s, this method has been used in more studies at a significantly lower cost. The sequencing results are compared to the 16S rRNA database to classify each sequencing result and determine the composition and relative abundance of all microorganisms in the community (15, 72).

Despite these advances, 16S rRNA gene sequencing has its limitations (1). PCR, which is used to amplify DNA from an entire community, introduces bias into the analysis, as some microorganisms are amplified more efficiently than others. Additionally, if a universal primer is not able to amplify the 16S rRNA gene of a particular microorganism, that microorganism will be missing from the sequencing data. 16S rRNA gene databases also present a limitation, as only microorganisms that have been deposited in these databases will be matched when mapping sequencing reads. Finally, the specific chosen amplicon that covers the targeted region of the 16S rRNA gene may affect the ability to detect certain microorganisms (102). Consequently, there may be several blind spots where certain microorganisms in the community may not be detected or may be misclassified due to limitations of universal primers or 16S rRNA gene databases. Nevertheless, this method provides a cost-effective analysis of the entire vaginal bacterial community, achieving a breadth of community analysis that is not possible with individual PCR assays. This is a powerful tool for researchers studying the vaginal microbiota and the pathogenesis of bacterial vaginosis (32, 72, 81, 83), but it is not commonly used in clinical practice because a cost-effective method requires testing several hundred samples, and the time required to perform these tests would delay patient treatment too much to provide clinical benefit.

As sequencing costs have rapidly decreased, a more comprehensive approach to sequencing bacterial communities has become more common. The use of shotgun metagenomic sequencing (SMS) allows for the sequencing of all DNA from the vaginal microbiota (23). The DNA is fragmented into small pieces and sequenced using high-throughput sequencing technology. This method bypasses the initial PCR using universal primers, eliminating this error. It also ensures that microorganisms that do not match the universal primers well are not missed, as the entire DNA is sequenced. While 16S rRNA gene sequencing provides a simple census of bacteria, SMS sequences the entire DNA and allows for the assessment of not only the identity of all bacteria present in the community, but also the complete DNA sequences of these bacteria. However, this requires much more sequencing effort than 16S rRNA gene sequencing, and therefore the cost of SMS is often more than 10 times higher per sample (105). The amount of additional cost is determined by the desired amount of sequencing for each sample. Increasing the amount of sequencing for each sample will provide a more complete picture of the microorganisms present in the community, as well as their functional potential.

In addition to its higher cost, SMS has other limitations. Since a specific gene is not amplified by PCR from the entire DNA, as is done in 16S rRNA gene sequencing, most of the sequencing effort in SMS may be spent on sequencing the host DNA (105). For this reason, SMS was initially applied to environments rich in bacteria, such as microbial mats (53) and the gut microbiome (56). The ratio of bacterial DNA to other DNA is a primary factor that determines the cost of SMS per sample (90). For example, if a given sample, such as a fecal sample, contains approximately 50% bacterial DNA, 600 GB of sequencing performed on a high-throughput sequencer will yield approximately 300 GB of bacterial sequence reads. On the other hand, if an eye swab contains about 1% bacterial DNA compared to 99% other DNA (host, etc.), then the same cost of 600 GB of high-throughput sequencing will only yield about 6 GB of bacterial DNA. Therefore, to achieve coverage of 300 GB of bacterial sequencing reads on eye swabs, you would need to pay 50 times more for sequencing and purchase 30,000 GB of sequencing. This may be economically prohibitive and a major driver in the adoption of SMS for high bacterial burden community analysis (90). However, as sequencing costs decrease, it may become more feasible to use SMS for low bacterial burden samples. Providing a clinically relevant interpretation of SMS data is currently a challenging task, given the ongoing uncertainty in the etiology and pathogenesis of BV, as well as the limited availability of the equipment and expertise necessary to obtain and analyze this data. An interpretable SMS software would be beneficial for providing clinicians with practical results.

A characteristic feature of BV is the presence of key cells, one of the Amsel criteria (5). Although this has been a known feature of BV for decades, it was not until 2005 that Swidsinski et al. confirmed that the key cell was a vaginal epithelial cell covered with an adhesive bacterial biofilm (95). It is now well established that BV is a biofilm infection. The biofilm is primarily composed of Gardnerella spp., although it is polymicrobial (85). The ability to accurately detect the presence of this polymicrobial biofilm is a highly specific marker for the diagnosis of BV (84). To achieve this goal, FISH is a promising probe-based technique, as it combines visual information from microscopy with histochemical methods and the specificity provided by molecular probes (70).

Traditionally, the use of FISH for bacterial identification is based on the hybridization of a synthetic DNA oligomer linked to a fluorophore that is complementary to the target 16S RNA sequence (3). FISH requires sample fixation, which improves the permeability of the bacterial cell wall, before the hybridization step, which typically occurs at temperatures ranging from 35 to 60°C, depending on the probe sequence (27). The sample is then observed using a fluorescence light microscope. Although this method can be very sensitive and specific, it is more labor-intensive and less sensitive than qPCR (33). However, unlike NAAT, FISH does not require the extraction and amplification of target biological material or controls for absolute quantification of bacteria in biological samples (62). It is important that FISH can be combined with flow cytometry for automated high-resolution analysis of mixed microbial populations (32). A possible strategy for high-throughput diagnostics would be to first analyze samples using flow cytometry. Once a large number of target species have been detected, the sample can be used for direct visualization using fluorescence microscopy.

The use of FISH for BV diagnosis has been shown in several studies. The first was conducted by Swidsinski et al. using 38 genus- and species-specific DNA-based probes, including a new probe targeting Gardnerella spp. (95). Although this was a relatively small study with 3 groups of 20 women each, the results confirmed that Gardnerella spp. was the predominant bacterial species in samples obtained from women with BV, while Lactobacillus spp.

were the main components in samples from healthy premenopausal women. Moreover, Gardnerella spp. dominated the BV biofilm and were only found in BV cases, while only a small amount of scattered Gardnerella spp. was found in a few healthy controls. It is important to note that BV biofilms included other species in addition to Gardnerella spp., but only at very low concentrations (95). The central role of Gardnerella spp. in BV biofilms has been confirmed by numerous subsequent studies. In a subsequent study by Swidsinski et al., Gardnerella spp. biofilms were only found in women with BV who were scheduled for curettage or laparoscopic salpingectomy (98). More recently, in a study involving 196 women and using 2 multiplex DNA probe assays, Jung et al. confirmed that biofilms dominated by Gardnerella spp. were present in women with BV, while Lactobacillus spp. dominated the vaginal microbiota of healthy control groups (52). A more recent study of 60 pregnant women found that, in addition to Gardnerella spp., F. vaginae and Sneathia spp. were the main components of BV biofilm (89). In an additional study of 500 vaginal samples, Gardnerella spp. and F. vaginae were the most frequently represented species in BV biofilm (94).

Specific Gardnerella spp. DNA-based probes have also been used to assess the presence and significance of Gardnerella in other diseases, such as inflammatory bowel disease (83). Other target species were also considered. Srinivasan et al. compared the prevalence of two curved gram-negative bacilli, Mobiluncus spp. and BVAB1 (recently renamed "Candidatus Lachnocurva vaginae" (47)), using DNA-based FISH probes in women with BV or normal vaginal microbiota, comparing these results with qPCR and metagenomic analyses to determine whether the Mobiluncus spp. observed in Gram-stained vaginal samples are actually BVAB1 ("Candidatus Lachnocurva vaginae") (93).

Over the years, several improvements have been made to FISH technology (103). A major breakthrough was the replacement of DNA probes with peptide-nucleic acid (PNA) probes, which significantly improved the permeabilization steps and increased the sensitivity and specificity of the FISH method (75). PNA probes are similar to DNA probes, but they have an uncharged polyamide backbone instead of a sugar-phosphate backbone (77). This leads to stronger hybridization due to the lack of electrostatic repulsion between the DNA probe and the negatively charged sugar-phosphate backbone of the target (78). The first DNA probes developed for BVAB studies were highly specific for Gardnerella spp. (64) and Lactobacillus spp. (63). Machado et al. They then demonstrated the high specificity and accuracy of the duplex PNA approach for diagnosing BV in clinical samples (65) according to the Isone-Hay criteria (49). Hardy and colleagues used a different approach by developing a PNA probe specific for F. vaginae (41). While the dual detection of Gardnerella spp. and F. vaginae serves as a highly specific marker for BV (68), the F. vaginae probe itself had a lower sensitivity (~67%) (41). In an attempt to improve this sensitivity, a more reliable F. vaginae probe has recently been developed that can be used in multiplex analysis together with the Gardnerella spp. probe (41). Although the data show an in vitro sensitivity and specificity of 100% and 99.9%, respectively, the actual effectiveness of this probe in vaginal samples from women with BV has not yet been determined.

Although there is still no commercial FISH-based assay for the diagnosis of BV, the success of FISH as a diagnostic tool for detecting bacterial infections has been well-established over decades of research, and FISH has been approved by the US FDA and the European Medicines Agency (EMA) for use in microbiological clinical analysis (2). Known existing clinical applications of FISH include the diagnosis of bloodstream infections (109, 55, 16), infectious endocarditis (89), and gastrointestinal infections (90), among others. The limitations of FISH are that it can be expensive, and specialized laboratory equipment and expertise are required to perform this technique. Nevertheless, the results are reliable and can be obtained in just 6 hours, and bacterial microorganisms of any type can be detected in clinical samples.

Treatment

Clinical treatment of BV has not advanced much in the last two decades. First-line antibiotic therapy has shown 70-80% cure after four weeks of treatment (110); however, a high relapse rate has been observed over a twelve-month period, reaching 40-50% (10). Therefore, new strategies have been explored to improve treatment efficacy, including the use of probiotics and prebiotics, acidifying agents, antiseptics, herbal products, vaginal microbiota transplantation, and phage endolysins.

Probiotics are live microorganisms that, when consumed in sufficient quantities, benefit human health, strengthen the immune system, protect against pathogens, and promote recovery.

Treatment is carried out in several stages. In the first stage, it is necessary to suppress the opportunistic microflora, and the drugs of choice are metronidazole and clindamycin. Alternative antibiotics include tinidazole and seknidazole. The second stage is aimed at restoring the normal microflora and includes the use of probiotics and eubiotics with strains of lacto- and bifidobacteria (L. rhamnosus, reuteri, and fermentus).

Prebiotics

Prebiotics are a source of nutrients for certain species and promote the growth of beneficial microorganisms. This is another alternative that has been studied as part of the treatment for BV (Vieira-Baptista et al., 2022). Collins and his colleagues evaluated a set of prebiotics, namely lactitol, lactulose, raffinose, and oligofructose, and their ability to stimulate Lactobacillus and BV-associated bacteria. Lactulose has been found to be the most promising prebiotic, as it specifically stimulates the growth of lactobacilli and does not affect the bacteria associated with bacterial vaginosis (Collins et al., 2018). The use of maltose gel has been tested on the vaginal microbiota of animals (rhesus monkeys), which is normally colonized by anaerobic bacteria associated with bacterial vaginosis. The prebiotic maltotex stimulated the growth of lactobacilli, which led to the suppression of bacteria associated with bacterial vaginosis in the vagina (Zhang et al., 2020).

Lactoferrin is another prebiotic that has been studied. Otsuki and Imai reported the use of lactoferrin (vaginal suppositories 150 mg/day and oral tablets 700 mg/day) in six women with a history of miscarriage or premature birth and refractory bacterial vaginosis. After a month of lactoferrin supplementation, lactobacilli became dominant in the vaginal microbiota, and in pregnant women, childbirth was normal and uncomplicated (Otsuki and Imai, 2017). Prebiotics have also been tested in combination with antibiotics. More recently, the antimicrobial activity of bovine lactoferrin, either alone or in combination with metronidazole or clindamycin, has been studied against G. vaginalis isolates. The results showed that lactoferrin inhibits the growth of Gardnerella depending on the dose, and the combination with clindamycin leads to a synergistic effect (Pino et al., 2022).

A group of patients with bacterial vaginosis received treatment with metronidazole (250 mg tablets, 3 times a day) in combination with a prebiotic vaginal gel (5 mg vaginal gel per day) for 7 days. The study found that the symptoms of infection were less severe in the treated group than in the group that did not receive the prebiotic gel (Hakimi et al., 2018). A recent review analyzed studies that used probiotics/prebiotics in combination with antibiotics to treat bacterial vaginosis. The review concluded that combination therapy was more effective in preventing recurrent bacterial vaginosis than using antibiotics alone (Afifirad et al., 2022).

Lactic acid is one of the studied treatment options for BV due to its antimicrobial activity against BV-associated bacteria and its ability to restore optimal conditions for lactobacilli. Several over-the-counter products are available, although the use of these products is not recommended in the guidelines (Plummer, Bradshaw, et al., 2021). According to an early study, the use of lactic acid gel (225 mg for 7 days) was as effective as oral

metronidazole (500 mg twice a day for 7 days) in treating patients with bacterial vaginosis. Moreover, the combination of gel with lactic acid and metronidazole showed better results than the use of metronidazole alone, and contributed to the colonization of the vaginal microbiome by lactobacilli (Decena et al., 2006). After treatment with metronidazole (2 g orally, once), lactic acid pessaries were tested to evaluate their effectiveness in combating biofilm caused by group B bacteria. After treatment with metronidazole, most women with group B bacteria experienced symptom resolution, and only 27.3% of them had biofilm detected at a follow-up visit (7-28 days) when they began treatment with lactic acid for 3 weeks (twice a week). At the third visit, the percentage of patients with biofilm decreased to 18.2%, but at the fourth visit (at the end of treatment), it increased to 36.4%, and the recurrence rate was high (Gottschick et al., 2017). More recently, Armstrong-Buissinette and colleagues conducted a large, controlled trial to compare the effectiveness of intravaginal lactic acid gel (5 mL, once daily for 7 days) and oral metronidazole (400 mg, twice daily for 7 days) in the treatment of bacterial vaginosis. Data on primary outcomes were available for 409 participants (204 received metronidazole, 205 received lactic acid gel). The resolution of BV symptoms at week 2 was higher with metronidazole (70%) than with lactic acid gel (47%). Similarly, microbiological resolution of BV at week 2 was higher with metronidazole (59/77, 77%) than with lactic acid gel (31/73, 42%), although more side effects were reported in the metronidazole group. However, follow-up for 6 months after treatment showed that the recurrence rate was similar for different treatment methods in participants who initially improved (metronidazole: 51/72, 71%; lactic acid gel: 32/46, 70%) (Armstrong-Buissere et al., 2022).

Boric acid has been used for decades to treat vaginal infections, including bacterial vaginosis, but there is still limited evidence for its use in this context (Powell et al., 2019). In one of the first clinical studies on the treatment of recurrent bacterial vaginosis, patients were prescribed oral nitroimidazole for 7 days, followed by boric acid for 21 days (intravaginal, 600 mg per day), and metronidazole gel twice a week for 16 weeks if remission was achieved. The study found that the cure rate was 87% after 12 weeks, but decreased to 65% after 28 weeks of treatment (Reichman et al., 2009). More recently, Marrazzo and colleagues used TOL-463 boric acid-based vaginal gel (2 g as a tampon or 5 g as a gel, once daily for 7 days) to treat bacterial vaginosis. They reported 50-59% early clinical cure rates and found this strategy to be effective and safe for treating bacterial vaginosis (Marrazzo et al., 2019).

Recently, a combination of traditional antibiotic therapy with boric acid was tested for the treatment of recurrent bacterial vaginosis. The treatment regimen included oral administration of nitroimidazole (500 mg orally) twice daily for 7 days, accompanied by vaginal administration of boric acid at a dose of 600 mg daily for 30 days, followed by vaginal administration of 0.75% metronidazole gel twice weekly for 5 months. After 30 days of treatment, only one patient still had symptoms, and she was diagnosed with refractory bacterial vaginosis. After 5 months of maintenance therapy, 21 of the 69 patients developed bacterial vaginosis, and 9 of the 29 women developed bacterial vaginosis 6 months after the therapy was discontinued. In general, 20 women did not develop bacterial vaginosis during the year (Surapaneni et al., 2021).

A recent study used acid-electrolized water containing 6% hydrochloric acid against Gardnerella spp. This new product demonstrated an antibacterial effect, inhibiting the growth of Gardnerella and exhibiting higher antimicrobial activity than metronidazole. Additionally, it had a minimal impact on L. acidophilus. Furthermore, vaginal samples were collected from women with bacterial vaginosis, and the new product was able to completely eliminate the viability of microorganisms in the cultured samples (Zhao et al., 2022).

Antiseptics.

Antiseptics include a large group of different compounds such as benzydamine (Boselli et al., 2012), chlorhexidine (Mirzaeei et al., 2021), dequalinium chloride (Mendling et al., 2016), octenidine (Swidsinski et al., 2015), polyhexamethylene biguanide (Koban et al., 2012), Povidone-iodine (Wewalka et al., 2002), which has been tested against vaginal infections for several years. They have a broad spectrum of action and generally destroy the cell membrane. Very little is known about cases of resistance to these compounds. Chlorhexidine has recently been used in clinical trials to treat patients with bacterial vaginosis in comparison with metronidazole (250 mg tablets, twice a day) for 5 days. Patients who received vaginal gel with chlorhexidine were more satisfied with the treatment than those who took metronidazole orally. Symptoms improved by 100% in both groups, but more patients who took chlorhexidine reported side effects (Mirzaeei et al., 2021).

Decvalinium chloride is one of the most studied antiseptics for the treatment of bacterial vaginosis. It has demonstrated good efficacy in the treatment of this condition (Mendling et al., 2016). Weissenbacher and colleagues compared the efficacy of treating bacterial vaginosis with dequivalinium chloride (10 mg vaginal tablets) and vaginal clindamycin cream (2%) in a randomized clinical trial. They reported that these two different treatment methods were equally effective, and the rates of clinical cure one week after treatment were similar (Weissenbacher et al., 2012).

More recently, a study showed that dequilin chloride can destroy Gardnerella biofilms and reduce the metabolism and biomass of Gardnerella biofilms (Gaspard et al., 2021). A recent study reported the use of dequilin chloride (100 mg vaginal tablets) for 6 days in 573 patients diagnosed with bacterial vaginosis. After treatment, about 85% of patients reported relief of symptoms within 4-6 weeks (Antoni Vives et al., 2022).

A comparison of the effectiveness of octenidine hydrochloride/phenoxyethanol and metronidazole (500 mg vaginal tablets) treatment for 7 days showed that both treatments had similar results, and a longer course of octenidine treatment (7 days vs. 14 days) resulted in a significant increase in the percentage of cured patients (Mikich and Budakov, 2010). However, in a later study, Swidzinski and colleagues demonstrated that despite the high cure rates after 7 days of treatment with octenidine and the ability to eliminate Gardnerella from the biofilm in patients with bacterial vaginosis, the recurrence rate of infection was approximately 66% after 6 months, and the biofilm was detected again. Moreover, repeated and prolonged treatment with octenidine led to increased bacterial resistance (Swidzinski et al., 2015).

Natural plant-based products.

Natural products have been used for several years to combat pathogenic microorganisms that cause various infections (Kim et al., 2022). Natural plant-based products are a growing and effective approach to treating bacterial vaginosis, as their mechanism of action can prevent the development of antimicrobial resistance in bacteria, which is a significant advantage over antibiotics (Palmeira-de-Oliveira et al., 2013).

In early 1991, Blackwell reported the first successful therapeutic use of tea tree oil for the treatment of bacterial vaginosis (Blackwell, 1991). Since then, several essential oils and their main components have been studied for their effectiveness in treating vaginal infections (Falconi-McCahill, 2019). One of the first studies demonstrating the antibiofilm potential of thymol, a small hydrophobic molecule found in thyme essential oil, showed its inhibitory effect in vitro on both newly formed and mature biofilms of Gardnerella spp. (Braga et al., 2010). Artemisia princeps Pamp. essential oil has a significant impact on the suppression of Gardnerella growth, as well as some of its main components (Trin et al., 2011). More recently, the effect of individual compounds from T. capitata on Gardnerella biofilms has been studied, showing high activity in inhibiting the cultivability of biofilms (Sousa et al., 2022). T. capitata oil has also been tested on a polymicrobial biofilm of six

species associated with bacterial vaginosis, showing a reduction in biofilm biomass and cultivability (Rosca, Castro, Sousa, França, Cavaleiro, et al., 2022).

Myrtus communis, Berberis vulgaris, or vaginal gel with metronidazole were used in 120 women with bacterial vaginosis. The groups that received M. communis or B. vulgaris had higher efficacy than the group that received metronidazole, and no recurrences were reported, while 30% of women in the metronidazole group had recurrences (Masudi et al., 2016). In a randomized trial, 80 women with bacterial vaginosis received treatment with metronidazole or vaginal cream Calendula officinalis . After treatment, no symptoms of bacterial vaginosis and no side effects were found in both groups (Pajohide et al., 2018).

The effectiveness of combining essential oils with antibiotics in the treatment of bacterial vaginosis was also studied. M. communis extract combined with metronidazole was used as a vaginal gel to treat women with bacterial vaginosis. Treatment with a combination of metronidazole and extract was effective in treating bacterial vaginosis, and patients did not experience reinfection within 3 weeks after treatment, although 12% of patients who received only metronidazole experienced reinfection (Masudi et al., 2017).

Taken together, these studies highlight the importance of studying essential oils and their main components as alternative treatments for bacterial vaginosis, and they also support the idea that bacteria can interact synergistically when cultured together, increasing resistance to antimicrobial therapy and causing frequent recurrences of bacterial vaginosis.

Vaginal microbiota transplantation method.

Vaginal microbiota transplantation (VMT) is another new and promising approach to treating dysbiosis, in which researchers aim to “restore” the vaginal microbiome to a healthy state by directly inoculating vaginal secretions from a healthy donor into the vagina of a woman with BV (DeLong, Zulfiqar, et al., 2019; Lev-Sagie et al., 2019). Recently, important results were published from the first research study involving BMT intervention in symptomatic, refractory, and recurrent BV (Levsagie et al., 2019). Lev-Sagy et al. selected five patients with recurrent BV for VMT procedures; four of them experienced complete long-term remission during follow-up (5-21 months after VMT), with a restored vaginal microbiome dominated by lactobacilli, significant improvement in symptoms, and normalization of the Amsel criteria. The authors also noted that multiple VMT sessions may be required to achieve a lasting clinical effect. Safety is an important aspect of VMT, given the risk of infection with infectious microorganisms or pathogens during the treatment of diseases, especially in immunocompromised recipients. Other works address how research in virtual medical therapy should be conducted, and some protocols are available regarding the design, methodology, and reproducibility of research in virtual medical therapy (DeLong, Bensouda, et al., 2019; Yockey, et al., 2022).

Research on probiotics.

Interest in using probiotics to treat bacterial vaginosis has been around for a long time. Three decades ago, Hallen and his colleagues conducted the first study that aimed to treat bacterial vaginosis with probiotics alone. Women were randomly assigned to groups for treatment with L. acidophilus, and 57% of women experienced significant improvements in their vaginal swabs (Hallen et al., 1992). A recent in vitro study showed that Lactobacillus can inhibit the formation of Gardnerella biofilm and reduce its biomass. The percentage reduction in biofilm formation was higher when using L. rhamnosus (32.7% ± 1.9% and 29.4% ± 2.7%) than when using L. casei (12.6% ± 0.7% and 0.5% ± 1.6%) for biofilm formation in 24 and 48 hours, respectively (He et al., 2021).

On the other hand, the first study that used oral probiotics was conducted in 2012. The authors reported a significant decrease in vaginal pH after taking oral probiotic yogurt (100 g twice a day for 1 week) compared to taking oral clindamycin (300 mg twice a day for 1 week). Since 80% of patients in the probiotic group and 84% of patients in the clindamycin group experienced complete resolution of symptoms, Hantoushzadeh and his colleagues concluded that probiotic and antibiotic treatments were equally effective (Hantoushzadeh et al., 2012). More recently, a randomized controlled cross-over trial was conducted in which patients took one capsule containing three sub-strains of L. crispatus (109 CFU/strain, once daily for 1 week). There was a significant reduction in the Newgent score and the number of Gardnerella spp. (Rostock et al., 2019). However, the effectiveness of probiotics in treating bacterial vaginosis remains controversial. A study in which the recommended first-line therapy, oral metronidazole (400 mg twice daily for 7 days), was combined with vaginal application of either 2% clindamycin cream (one applicator for 7 days) or vaginal probiotic L. acidophilus (1 × 107 CFU, one pessary for 12 days) did not reduce the recurrence rate of bacterial vaginosis over 6 months (Bradshaw et al., 2012). A new search study evaluated the use of vaginal capsules containing L. gasseri and L. rhamnosus at a concentration of 1 × 108 CFU/capsule. Patients with bacterial vaginosis received either oral antibiotics (cefixime, doxycycline, and metronidazole) for 7 days, or a combination of antibiotics followed by probiotics once a day for 30 days, and then once a week until day 190. The results showed that women who received a combination of antibiotics and probiotics had a higher number of introduced Lactobacillus species in their vaginal microbiome; however, there were no differences in the recurrence rate of BV at 6 months between women who received only antibiotics or antibiotics and probiotics (Marcotte et al., 2019).

From an immune perspective.

BV is not characterized as a neutrophilic disease (79). Leukocytes in the vagina are rare in BV, except in cases of concomitant vaginal (Trichomonas vaginalis or vulvovaginal candidiasis) and/or cervical infection (e.g., C. trachomatis) (39). BV is also rarely associated with pain, redness, or swelling, which are characteristic of extensive tissue inflammation (79). However, studies have shown increased levels of cytokines and chemokines (e.g., IL-1β, TNFα, IL-6, and IL-8) in the vaginal secretions of women with bacterial vaginosis (43). Early colonizers, such as G. vaginalis and P. bivia, can actively suppress the body's inflammatory response in the vaginal epithelium, avoiding the effects of the immune system during the formation of a biofilm in bacterial vaginosis. This is supported by a study in mice, where P. bivia alone or in combination with G. vaginalis did not cause increased histological inflammation in vaginal tissues (40). In contrast, secondary colonizers of the BV biofilm (e.g., A. vaginae and other BVAB) can stimulate the host immune response in vaginal epithelial cells and cause symptoms (e.g., vaginal discharge and odor) and signs (e.g., homogeneous white vaginal discharge) of BV (73). BVAB-derived metabolites, including biogenic amines, are associated with these BV symptoms (78, 92).

Individual types of microorganisms as possible causes of BV.

Since it is clear from historical studies of BV that no single type of bacteria is present in all cases of BV by any definition, it is necessary to consider in detail the interactions between organisms that coexist on the human body (19). Despite the fact that numerous studies have revealed a link between bacterial vaginosis and the presence of a number of bacterial genera and species, the role of these bacteria in the etiology and pathogenesis of the disease remains unclear. The complexity and variability of the vaginal microflora make it difficult to simply and definitively determine which organisms are pathogenic. The combination of molecular and cultural methods applied to complex clinical samples and the creation of physiological models that allow for the analysis of the body's immune responses to individual microbes have provided valuable new information about the key characteristics of specific bacterial species that cause bacterial vaginosis.

The ability of G. vaginalis to attach to vaginal epithelial cells provides a basis for the formation of a biofilm and for other BV-specific bacteria, such as Atopobium vaginae, to become established in this biofilm. G. vaginalis and A. vaginae have been found together in vaginal biofilms and in association with the presence of key cells. Biofilm formation plays a key role in the development of the disease, as it increases resistance to antibiotics and the body's immune defenses, leading to a chronic course of the disease and/or relapses. Svidzinsky et al. Using fluorescent in situ hybridization (FISH), it was shown that a characteristic dense biofilm consisting of fused or discontinuous layers was attached to at least 50% of the intact epithelial surface in 90% of vaginal biopsy samples from patients with bacterial vaginosis, compared with 10% in healthy women from the control group (96). vaginalis was the predominant bacterium in these biofilms, followed by A. vaginae, which was present in 80% of biofilms and accounted for up to 40% of their mass. G. vaginalis biofilms found in women with bacterial vaginosis are resistant to higher concentrations of hydrogen peroxide and lactic acid (18). Atopobium resistance The use of metronidazole and its association with G. vaginalis biofilms may explain the high recurrence rate of bacterial vaginosis. The production of amines leads to an increase in pH and promotes the growth of other anaerobes associated with bacterial vaginosis. Finally, G. vaginalis peptidases can affect the protein-rich vaginal environment, releasing peptides and amino acids that, in turn, stimulate the growth of bacteria and provide them with the nutrients necessary for the growth and co-dependence of other organisms associated with bacterial vaginosis. Interestingly, viable G. vaginalis bacteria can be engulfed by vaginal epithelial cells, involving active reorganization of the epithelial cytoskeleton, and this engulfment activates factors that promote the attachment of other pathogenic bacteria, such as E. coli (66). Thus, the multiple properties of G. vaginalis, including its biofilm formation, metabolic activity, engulfment by epithelial cells, and modulation of host immunity, as described below, may contribute to the diversity and survival of the bacterial vaginosis-associated microbiota and its resistance to therapy. Resistance of bacterial vaginosis to therapy is associated with a higher level of G. vaginalis after a standard 7-day course of treatment with metronidazole (101).

Clinical studies have confirmed a significant association between G. vaginalis and impaired vaginal immunity. Hedges and his colleagues found that women with the highest number of Gardnerella or Prevotella morphotypes (described below) present in their vaginal swabs (>30 per field of view under high-power microscopy) had elevated levels of IL-1β in their vaginas. Using culture methods, Anderson and his colleagues (7) established a link between the presence of G. vaginalis in vaginal swabs from low-risk pregnant women and elevated levels of IL-1β, gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and granulocyte-macrophage colony-stimulating factor (GM-CSF) in the cervix.

Similarly, genomic PCR analysis of vaginal smears showed that G. vaginalis is involved in increasing cervicovaginal levels of IL-1b and other pro-inflammatory cytokines, for example IL-1a, IL-6, IL-8 and IL-12p70, although some immunosuppressive effects were also observed, for example, a decrease in interferon-gamma levels.inducible protein 10 (IP-10) and SLPI (42). G. vaginalis was also one of the six dominant species identified by 16S rRNA gene sequencing in a type of microbiota community characterized by higher cervicovaginal levels of IL-1a, IL-β, IL-8, TNF-α, IFN-γ and IL-10 in young South Africans at risk of HIV infection (6). A recent analysis of the 16S rRNA gene confirmed that a high concentration of gardnerella, in particular, combined with a low concentration of lactobacilli, may contribute to an increased risk of premature birth (20). Thus, there is strong evidence that G. vaginalis affects host immunity and the pathogenesis of bacterial vaginosis.

vaginae was detected by PCR in 96% of women with bacterial vaginosis and only in 12-19% of women without it (12, 28, 104). However, Menard et al. found A. vaginae in 69% of samples taken from women without bacterial vaginosis, suggesting that the detection of A. vaginae alone is not a reliable indicator of bacterial vaginosis. Nevertheless, their results showed that the quantification of A. vaginae bacteria is a good predictor, as higher levels were found in BV-positive samples (69). A. vaginae has been associated with three of the four Amsel clinical criteria, including vaginal discharge, elevated pH, and the presence of key cells (91). A. vaginae and G. vaginalis have been shown to be present in 78-96% of BV samples, compared to 5-10% of normal flora samples (69, 112). An analysis of the composition and structural organization of the biofilm attached to the vaginal mucosa in patients with bacterial vaginosis showed that A. vaginae was present in 70% of the samples, accounting for 1 to 40% of the film's mass (96). The association of A. vaginae with biofilm formation and resistance to metronidazole may explain the failure of treatment and the recurrence of bacterial vaginosis.

A. vaginae has become a powerful factor that causes inflammation and innate immune responses in the vaginal epithelium (21, 25-26, 60). It activates the main pro-inflammatory transcription factor NF-κB in cervicovaginal epithelial cells (26). Despite conflicting data on the effect on cytokines, such as IL-6 and TNF-α (21-22), all studies conducted to date have shown that A. vaginae significantly increases the expression of chemokines in vaginal and/or cervical epithelial cells, including IL-8 (22, 25, 59), MIP-3α (CCL20) (21), and RANTES (CCL5) (25). It has been shown that, like G. vaginalis, A. vaginae interacts with the Trichomonas vaginalis virus and can penetrate into vaginal epithelial cells, where it remains viable even after antibiotic treatment, possibly receiving protection from competition with protozoan parasites or other vaginal microorganisms (25). In clinical studies, the detection of A. vaginae in vaginal swabs was correlated with higher levels of the same inflammatory mediators associated with G. vaginalis (42), and Atopobium was one of the most prevalent taxa in microbiota communities with the highest levels of cervicovaginal inflammatory mediators, according to a study involving women from South Africa (6).

Conclusions

The vaginal flora of healthy women, which is dominated by lactobacilli that protect against infections, is less diverse than that of patients with bacterial vaginosis, whose microbiota is more diverse and contains many obligately anaerobic and unculturable species. This polymicrobial disease is accompanied by relatively simple clinical symptoms that do not occur in all women, which makes it difficult to determine its etiology. Treatment regimens that include only probiotics are safe and may have short- and long-term positive effects in the treatment of bacterial vaginosis. The results of using probiotics after antibiotics, depending on ethnicity, deserve further study. Further research on the vaginal bacterial community is necessary to study antibiotic resistance and develop more effective alternative therapeutic strategies to reduce the symptoms of bacterial vaginosis and its associated complications. In general, unraveling the mystery of the pathogenesis of bacterial vaginosis is key to the prevention and treatment of this public health problem.

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