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Application of Metabonomics Methods in Determination of Metabolites of Vaginal Microbiota

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Abstract

The bacterial composition of the vaginal microbiome is thought to be connected to women's health and disease status. Vaginal microbiome is dynamic, with life-related shifts in components diseases, such as pregnancy, menstruation, bacterial vaginosis, and HPV infection. Understanding the microecology of the reproductive tract, people started to pay more attention to eco-diagnosis and eco-therapy to understand and adjust the microbiota. Moreover, the new eco-therapy improves the condition of the disease by adjusting the microbiota. Metabolites are an essential component of the vaginal microenvironment. The differences between some metabolites are to the functional heterogeneity of the vaginal microbiota. In addition, the metabonomics method can conduct quantitative and qualitative analysis of metabolites. Inspired by this, we can use metabonomics methods to detect metabolites in the vagina, find their correlation with gynecological diseases, and provide an idea for future women to solve the "embarrassing" problem. This summarizes several common metabonomics methods used to measure vaginal metabolites in recent years, and uses common mass spectrometry, chromatography, and spectroscopy instruments to analyze metabolites of vaginal microbiota.

Keywords: Metabonomics; vagina; gynecological diseases;1H-NMR; LC-MS; GC-MS; MS; HPLC

Introduction

Vaginal microbiota plays a vital role in maintaining the function of the female reproductive tract and health, and in preventing sexually transmitted diseases and the colonization of genitourinary tract pathogens. Unlike most other parts of the human body, vaginal microbiome is highly dynamic and characterized by temporal perturbations, affected by sexual development, sexual intercourse, menstruation, hormone levels, and personal hygiene [1]. As a result of this dynamic change in the vagina, microorganisms and compositions may be unstable at different times, even for a particular woman. The development of high-throughput DNA sequencing technology has significantly enhanced the ability to study human microbiota. New genetic technology approaches used in previous studies, such as 16S rRNA gene sequencing, investigated the composition of the vaginal

microbiota in relation to various health and disease states [2-4]. The use of new generation molecular sequencing techniques has shown that the majority of vaginal bacterial communities are led by *Lactobacilli* [5,6]. Ravel et al. analyzed vaginal microbiota from non-pregnant, fertile, and asymptomatic groups and summarized the vaginal bacterial communities in five major categories of community-state types (CSTs).

Four CSTs were dominated by different species of *Lactobacillus* (: *L. crispatus*, CST I; *L. gasseri*, CST II; *L. iners*, CST III; and *L. jensenii*, CST V). The other communities (CST IV) were varied and formed by a large proportion of obligate anaerobic bacteria, including *Gardnerella, Atopobium, Prevotella spp*.., and other bacterial species [7]. The high risk of CST IV was relative to chronic HPV infections, sexually transmitted diseases (STDs), HIV infections, preterm birth, and adverse pregnancy outcomes such as post-abortion sepsis,; early, late, and recurrent abortions,; etc. [8-14].

As the understanding of the human microecosystem gradually deepened, people started to pay more attention to eco-diagnosis and eco-therapy to understand and adjust the microflora. Recent studies on the interaction between vaginal microecology and vaginal epidermal cells suggest that vaginal health can be maintained by improving the composition of the metabolites in the vagina. Further, many studies have shown that vaginal flora and its metabolites have a very significant indicator of whether women are suffering from gynecological diseases [3]. Unlike genotyping techniques that have been validated and consolidated in microbiological research, there is little knowledge on the use of metabonomics in microbial identification. Metabolites are essential component of the microenvironment of the reproductive tract. The differences between some metabolites are due to the functional heterogeneity of the vaginal microbiota. Flora metabolites may become a signal molecule for the diagnosis of gynecological diseases. Future research focused on genomics, proteomics, and metabonomics technologies may potentially have a significant impact on women's reproductive health and disease treatment.

Metabonomics is a basic and powerful tool. It can obtain various information on the health and disease status of the host, including the changes in bacterial types and the correlation of microenvironment, in order to fully understand the overall situation of growth and development or disease development. The following is a summary of the four methods of metabonomics to measure vaginal metabolites: proton nuclear magnetic resonance (1H-NMR), high-performance liquid chromatography (HPLC), mass spectrometry, gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (LC-MS).

Proton Nuclear Magnetic Resonance Spectroscopy

Metabonomics uses high-flux analysis methods such as magnetic resonance (1H-NMR) spectroscopy to analyze complex systems for accurate and sensitive identification of metabolites produced by microorganisms and host cells. This tool has a great advantage in the quantitative and qualitative analysis of thousands of metabolites [15]. Foschi et al. used 1H-NMR spectroscopy to analyze the metabolic characteristics of Lactobacillus from 40 strains of various origins (gut, vagina, food, probiotics). The results identified a group of metabolites whose variations in concentration were correlated with the taxonomy, but also showed a high intra-species variability that did not allow for species-level identification. There can be important variations in the metabolic characteristics of related species, and several metabolic pathways and molecules are associated with particular species. Although 1H-NMR spectroscopy analysis cannot be proposed as a reliable tool for the identification of Lactobacillus species or strains, it can help to study the metabolites of different strains of *Lactobacillus*. Later, Camilla et al. described in detail the composition of vaginal metabolites based on 1H-NMR spectroscopy. The alteration in bacterial community that occurs during genital infection is a major change in the composition of vaginal metabolites, and the reduction of lactic acid is a typical sign of all pathological conditions. The data revealed that the vaginal secretions of bacterial vaginosis (BV) infected women had a higher content of biogenic amines and short-chain organic acids than normal women. In conclusion, based on the metabonomics analysis, we found that different vaginitis infections have unique vaginal metabolome fingerprints using 1H-NMR analysis [16]. In the future, the use of 1H-NMR spectroscopy to analyze metabolites of different strains can be used to evaluate the effect of intervention or treatment of vaginal infection.

At the same time, Parolin et al. used 1H-NMR spectroscopy to evaluate the metabolic profile of *Chlamydia trachomatis* (CT) infected women. Regarding the metabolome, 4-aminobutyrate showed significant different concentrations in asymptomatic (2.08×10−3±[0.21×10−2) and symptomatic (1.01×10−2±2.72×10−2) (P = 0.027).) CT women. In addition, vaginal swabs were collected and analyzed using 1H-NMR, which provides a new perspective for future metabonomics research [17].

Vitali et al. recruited vaginal fluid of affected patients and healthy controls to examine metabolites by 1H-NMR

spectroscopy. Furthermore, significant differences in the concentrations of 32 metabolites were observed between BV and non-BV women ($P < 0.05$). A molecular combination method was proposed to detect niacin, malonic acid, and acetate as a biomarker for BV infected women by combining qPCR and 1H-NMR spectroscopy [18].

High-Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is a powerful technique for the study of specific metabolites, including amino acids. HPLC has the advantages of providing fast, quantitative, and easy access to a variety of metabolites. In addition, it is highly adaptable since any compound that is soluble in liquid samples can be separated, identified, and quantified by an HPLC. Sungur et al. studied the inhibitory and immunomodulatory effects of extracellular polysaccharides (EPSs) of *L. gasseri* strain (from normal vagina) on the growth of cervical cancer cells (Hela) by HPLC [19]. Additionally, the monosaccharide composition of the L-EPSs produced by *L. gasseri* strains was determined by HPLC. In conclusion, diversity in sugar composition of EPS might contribute to properties of adhesion and proliferation. This indicates that the metabolites of vaginal flora may be used as targets for the treatment of gynecological diseases in the future. Subsequently, Welch et al. used HPLC to study the inhibitory effect, potential therapeutic role, for an analog of reutericyclin (glycerol monolaurate; GML), against microbial pathogens, including human immunodeficiency virus type 1 (HIV-1). The results showed that HPLC-purified reutericyclin and reutericyclin secreted by *Lactobacillus* inhibit HIV-1. These data emphasize the importance and protective nature of the normal vaginal flora during viral infections and provide insights into the antiviral mechanism of GML during HIV-1 infection and, more generally, to other enveloped viruses [20].

Polat et al. used HPLC to study the expression of vaginal metabolites in women who were diagnosed with a microbial invasion of the amniotic cavity of preterm women. The HPLC data showed that the concentrations of taurine, lysine, and cysteine in women with an amniotic cavity invasion were significantly higher than in women without infection. However, the ratio of vaginal glutamic acid, aspartic acid, and aspartic acid to aspartic acid in the vagina of infected women was significantly lower than that in uninfected women [21].

Mass Spectrometry (MS)

MS produces gas -phase ions directly from the sample for rapid and cost effective metabolic analysis of unretouched and complex biological materials, including bacteria. It does not require chromatography separation or sample preparation for direct processing and rapid analysis of complete biological fluids. BV is a common but highly enigmatic condition associated with adverse outcomes for women and their neonates. Srinivasan et al. used MS to compare the metabonomics characteristics of cervicovaginal lavage fluid obtained from 40 women with BV to profiles in 20 women without BV. In their study, there was a substantial difference of 62% of the metabolites between women with BV and without BV. Higher levels of the signaling eicosanoid 12-hydroxyeicosatetraenoic acid (12- HETE), a biomarker for inflammation, were noted in BV. *Lactobacillus crispatus* and *Lactobacillus jensenii* exhibited similar metabolic correlation patterns, which were distinct from the correlation patterns of BV-associated bacteria. Insights from this study provide opportunities for developing new diagnostic markers of BV and novel approaches to treatment or prevention of BV [22].

Prussian collected vaginal swabs from healthy individuals (n= = 41) and BV patients (n= 21), and directly analyzed the metabolites using desorption electrospray ionization -MS (DESI-MS). Further, swabs of BV patients were found to contain consistently high levels of amino acid metabolites, including polyaminooctane and methylamine. These compounds are the main cause of the fishy smell of the vagina, which proves the ability of these metabolites as biomarkers of BV metabolites. The DESI-MS method was also applied directly to bacterial biomass in order to confirm the ability to detect intact bacterial species from a swab. These results highlight the potential of direct swab analysis by using DESI-MS for a wide range of clinical applications, including rapid mucosal diagnostics for microbiology, immune responses, and biochemistry [23].

Gas Chromatography-MS (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS)

A multi-omic systems-based approach shows the metabolic markers of BV and insight into the disease. The most effective multi-omic systems- based method combined the advantages of HPLC and MS for metabolome analysis in recent years. LC-MS or GC-MS is a very versatile technology with a range of different LC(GC) or MS analyzers and analysis modes. The research of LC-MS based metabonomics is making rapid progress, and owing to the continuous improvement of histography and mass spectrometology ability, metabonomics has great potential in the field of biomedical science and has become a primary analytical technique for metabonomics [24]. In fact, not all substances maintain good stability in liquids. To detect more metabolites to find the corresponding biomarkers, most researchers would choose to use a link between the GC-MS and LC-MS to get better results.

Yeoman et al. analyzed 36 BV women with GC-MS, 176 different metabolites were found, 67 of which were significantly different. In addition to putrescine, cadaverine, 2-methyl-2-hydroxybutyric acid, etc.., most metabolites will be reduced in BV -infected women. The characteristic odor associated with BV was associated with increases in putrescine and cadaverine, both of which were relative to *Dialister spp*. Additional correlations were seen with, 2-methyl-2- hydroxybutanoic acid, *Mobiluncus spp*., diethylene glycol, and *Gardnerella spp*.

Thomas et al [25] used a GC-MS-based metabonomics approach to search for biomarkers that could serve as early indicators of spontaneous preterm birth (sPTB). Cervicovaginal fluids were collected at 20 weeks from women who were initially assessed as having a low risk of sPTB. GC-MS analysis revealed a total of 112 compounds derived from -cervical fluids that were classified mainly by metabolites such as amino, organic and fatty acids. The GC-MS methyl chloroformate derivatization method is more stable than other derivatization methods in the study. There was no significant correlation with low risk of sPTB in the statistical analysis.

Ghartey et al. were to investigate whether cervicovaginal (CV) metabolome was different in asymptomatic women for preterm birth (PTB) pathogenesis compared to term birth. A total of 313 biochemicals were analyzed and identified in CV fluid using GC-MS and ultra-performance liquid chromatography-tandem MS (UPLC-MS)-MS. Moreover, 82 biochemicals were different in 20–24 weeks(V1) for those destined to have PTB relative to term birth, while 48 were different at 24-–28 weeks (V2). Changes in the CV metabolome may be observed weeks prior to any clinical symptoms. Additionally, understanding the CV metabolome may be promising to unravel the pathogenesis of PTB and may provide novel biomarkers to identify the women most at risk [26].

Oliver et al. analyzed saliva and urine metabolomes using gas chromatography-time of flight MS (GC-TOF MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) lipidomics approaches for samples from mothers and their infants during the first year of life. The data showed that most women had either a simple community with one highly abundant species of Lactobacillus or a more diverse community characterized by a high abundance of Gardnerella. Here, sequencing and metabonomics techniques used to illustrate novel associations between vaginal microbes and metabolites during healthy pregnancy [27].

Borgogna et al. conducted a GC-MS and LC-MS analysis of metabolites on 39 subjects, including 13 HPV-negative [HPV (-)], 26 HPV-positive [LR-HPV (+)], and 14 high-risk HPV -positive [HR-HPV (+)]. They found that compared to women with LR-HPV, HR-HPV -infected women had lower concentrations of amino acids, lipids, and peptides in self-collected mid-vaginal swabs. The vaginal metabolome of HPV (+) women differed from HPV (-) women in terms of several metabolites, including biogenic amines, glutathiones, and lipid-related metabolites. If the temporal association between increased levels of reduced glutathione and oxidized glutathione and HPV incidence/persistence is confirmed in future studies, anti-oxidant therapies may be considered as non-surgical HPV management interventions [28].

Conclusion and Prospects

Vaginal microbial environments may be affected by many lifestyle and physiological changes, such as smoking, staying up late, menstrual cycle, pregnancy, menopause, and other hormonal changes. The correlation and interaction between different bacterial communities of the vagina play an important role in women's "health" and "disease". When female vaginal micro-environmental bacteria are in equilibrium, they can play a vital role in maintaining a healthy vaginal environment. After gynecological diseases occur, vaginal bacteria and vaginal metabolites will change dramatically. The observed increased vaginal ecosystem stability during pregnancy may play an important role in reducing susceptibility to elevated infections that may lead to intrauterine infections and subsequent preterm birth.

The summary of the research methods indicates above that the use of metabonomics to detect vaginal secretions or vaginal fluid is a potential development to understand the relationship between reproductive tract health and diseases. This methodology mays be used as diagnostic markers or treatment of gynecological inflammation in the future. The use of metabonomics to explore and analyze reproductive tract diseases has been a hotspot in recent years. Metabonomics can be used to explore and analyze vaginal metabolism of gynecological diseases such as bacterial vascular disease, premature women, HPV infection, and so on.

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