http://www.ijwhr.net
doi 10.15296/ijwhr.2020.68
Open Access Brief Report
IJWHR
International Journal of Women's Health and Reproduction Sciences
Vol. 8, No. 4, October 2020, 423–427
ISSN 2330-4456

Microbiome of the Uterus in Women With Unsuccessful in Vitro Fertilization Attempts

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Abstract

Objectives: This study aimed to investigate the peculiarities of the uterine microbiome in the case of infertility and repeated in vitro fertilization (IVF) failures. Accordingly, the microbiome of the uteri of 22 women with 2 or more IVF failures (first group) and 20 healthy women (second group) was analyzed in this study.

Materials and Methods: The samples of uterine microbiomes were collected 7 days after the luteinizing hormone elevation, which was determined by the urine test. All measures were taken to avoid sample contamination. Finally, the massively parallel sequencing of the 16S ribosomal RNA gene was done in every uterine sample.

Results: The analysis of the mean relative abundance of various microorganisms in the uterine microbiome showed that women with infertility had higher microbiological diversity and variability compared to healthy women. Eventually, the mean relative abundance of the *Lactobacillus* genus comprised 34.4% and 63.0% in the first and second groups, respectively.

Conclusion: In general, the presence of *Lactobacillus* in the uterine microbiome could be considered a favorable factor for good reproductive outcomes and successful IVF programs.

Keywords: Uterine microbiome, Infertility, *Lactobacillus*, IVF failure

Introduction

For nearly a century, the concept of Tissier (1) on the absolute sterility of the uterus was generally accepted in the scientific community. However, uterine peristalsis, which contributes to the capture of the sperm from the vagina, can accordingly contribute to the entry of vaginal microflora into the uterine cavity.

On the other hand, some studies proved the hematogenous pathway of bacteria entering various parts of the genital tract (2), either from the oral cavity (3) or the intestine (4). Other routes for the entry of microorganisms into the upper genital tract may include invasive procedures, embryo transfer procedures in in vitro fertilization (IVF) cycles, and the installation of intrauterine devices. With the advent of a new generation of sequencing technology, it has become possible to determine a wide range of microorganisms.

Considering the above-mentioned explanations, the purpose of this study was to reveal the difference between the uterine microbiomes of women with infertility and repeated IVF failures and those of normal healthy women with no burdened obstetrical or gynecological history.

Materials and Methods

Study Cohort

The study population included 42 women who were divided into two groups. All patients were recruited from the Department of Obstetrics and Gynecology №1, Rostov-on-Don State Medical University (Rostov-on-Don, Russia). The first group included 22 asymptomatic women with infertility and repeated IVF failure (2 or more attempts), who were in the age range of 20-46 years. The inclusion criteria were unexplained infertility, two and more unsuccessful attempts of IVF, the age range of 20-46 years, regular menstrual cycle (25-35 days), and normal anatomy of the uterus (proven by an ultrasound examination or hysteroscopy). On the other hand, the exclusion criteria included any pathology of the uterus, including leiomyomas, polyps, congenital malformations, and Asherman syndrome (intrauterine adhesions), as well as the usage of a contraceptive intrauterine device in the previous 6 months, any systematic inflammatory disease or severe extragenital pathology, and the recent use of systemic antibiotic therapy.

In addition, the second group included 20 healthy asymptomatic women aged 20-44 years. The inclusion criterion was the absence of burdened obstetrical or gynecological history, and the exclusion criteria were the same as those of the first group.

Sample Collection

The samples of uterine microbiomes were collected

Received 11 June 2020, **Accepted** 7 August 2020, **Available online** 17 October 2020

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Key messages

- Due to development of technologies of new generation sequencing, it is no doubt that uterine microbiome exists.
- The predominance of *Lactobacillus* in uterine microbiome can be considered as a predictor of favorable reproductive outcomes.
- High microbial diversity of uterine microbiome in women with repeated IVF failures is a predictor of failed implantation.

exactly 7 days following the luteinizing hormone surge, which was detected in the urine. A double-lumen embryo transfer catheter was used to avoid the contamination of the catheter by vaginal or cervical microbes. After the visualization of the cervix, a sterile swab with chlorhexidine solution was applied to remove extra cervical and vaginal mucus. Then, a double-sheathed catheter for embryo transfer was carefully inserted in the cervical canal, avoiding contact with the vaginal walls. Next, the inner catheter was advanced up to the fundus of the uterus and the endometrial fluid was collected accordingly. The inner catheter was then retracted into the outer catheter, and the whole system was drawn from the cervical canal. Finally, the endometrial fluid sample was placed in an Eppendorf tube with special transportation fluid ("Transportation fluid with a mucolytic", the Public Entity Central Research Institute of Epidemiology, Russia) and then kept in the medium at +4°C until DNA extraction.

Sequencing

DNA Extraction

The total DNA was extracted from the tissue samples using the "Rhibo Prep" kit (Public Entity Central Research Institute of Epidemiology, Russia) following the manufacturer's recommendations.

Library Preparation and Sequencing

In general, 16S DNA libraries were prepared according to the Illumina protocol "16S Metagenomic Sequencing Library Preparation" (Part № 15044223 Rev. B). Moreover, 5 ng of the total DNA was used to amplify the target 16S rRNA gene fragment and Illumina adapter sequences were added using the recommended primers for V3 and V4 regions. The primer sequences included 16S Amplicon polymerase chain reaction (PCR) forward primer = 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3', 16S Amplicon PCR reverse primer = 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATCC-3'.

Accordingly, 25 cycles of PCR were conducted using the KAPA HiFi HotStart ReadyMix (2X, Roche Diagnostics, Switzerland).

After the solid phase reversible immobilization bead purification of PCR products, 5 ng of the resulting amplicons were indexed using KAPA HiFi HotStart ReadyMix (2X, Roche Diagnostics, Switzerland) and the Nextera XT Index Kit (Illumina, USA).

Finally, 8 cycles of index PCR were conducted following the Illumina protocol, and libraries were sequenced on the Illumina MiSeq System.

Data Analysis

Sequencing data analysis was done using a custom bioinformatic pipeline implemented in R version 3.6 (R Core Team, 2014) and Python programming languages. At the first step of the pipeline, primer sequences were trimmed from the beginning of paired-end reads by read pairs not containing any discarded primer sequences. Next, trailing 25 base pairs were cropped from the end of each read as low-quality bases and the resulting data were processed using the DADA2 workflow for exact sequence variant identification (5). After the inference of exact sequence variants, forward and reverse reads were merged via concatenation, and the resulting sequences were used for naive Bayesian taxonomic classification (6) using the SILVA v132 reference database (7). Eventually, species assignment was done using an exact matching algorithm in DADA2 using SILVA v132 sequences, preprocessed accordingly using custom scripts.

Statistical Analysis

The groups were compared using the Mann-Whitney test, and differences were recognized as statistically significant with $P < 0.05$. Finally, calculations were performed in R (version 3.6, R Foundation for Statistical Computing, Vienna, Austria).

Results

The age of the patients ranged from 20 to 46 years in both clinical groups. In the clinical group I (asymptomatic women with infertility and repeated IVF failures), the average age of the patients was 31.8 ± 4.4 years. In clinical group II, patients' average age was 31.4 ± 4.9 years.

In addition, the body mass index (BMI) of all patients of both clinical groups ranged from 17 to 26, and that of the first and second groups was 21.9 ± 2.01 and 22.15 ± 2.2, respectively.

Further, the mean age of patients' menarche and the mean duration of the menstrual cycle were 13.27 ± 1.28 and 12.85 ± 1.50 years, as well as 28.18 ± 2.99 and 28 ± 2.71 days in the first and the second groups, respectively. Furthermore, mean menstrual duration comprised 5.59 ± 1.26 and 5.5 ± 1.39 days in the first and the second groups, respectively. Patients in both clinical groups were comparable in terms of age, BMI, age of menarche, duration of the menstrual cycle, and menstrual duration.

In general, 233 different microorganisms found in both groups were analyzed in this study. The comparison

of the relative abundance of microorganisms in the groups revealed significant differences in 14 of these microorganisms (Table 1).

Based on the results, higher relative abundance of *Lactobacillus iners* (37.2 [27.1, 44.6]), *Lactobacillus acidophilus* (7.24 [1.92, 12.6]), *Lactobacillus jensenii* (5.1 [3.59, 7.95]), *Lactobacillus crispatus* (7.38 [5.69, 10.2]), *Prevotella melaninogenica* (0 [0, 0.05]), *Bacteroides vulgatus* (0 [0, 0.05]), *Corynebacterium bouchesdurhonense* (0 [0, 0.05]), *Bacteroides caccae* (0 [0, 0.005]), and *Bifidobacterium adolescentis* (0 [0, 0.005]) was detected in the group of healthy asymptomatic women of reproductive age with no burdened obstetric or gynecological history. Women in the first and second groups had a higher relative abundance of *Gardnerella vaginalis* and *Bifidobacterium gallinarum*, respectively (Figure 1).

Moreover, higher relative abundance of *Methylobacterium aerolatum* (0 [0, 0.21]) and *Comamonas testosteroni* (0 [0, 1.16]) was revealed in the group of women with infertility and repeated IVF failures. These microorganisms are not considered to be part of the normal human microbiome although evidence exists about their involvement in appendicitis (8). Therefore, they are regarded as a sign of the possible contamination of the sample medium or the air in the operation room.

The analysis of the mean relative abundance of various microorganisms in the uterine microbiome showed that women with infertility (first group) had higher microbiological diversity and variability as compared to healthy women in the second group (Figure 2). Based on the obtained data, the mean relative abundance of the *Lactobacillus* genus comprised 34.4% and 63.0% in the first and second groups, respectively. Eventually, the presence of *Streptococcus* spp. and *Gardnerella vaginalis* in small concentrations (3% and 3%, respectively) was also a hallmark of infertility and repeated IVF failures.

Discussion

Our study had several limitations, one of which was the sample size. In general, 22 and 20 patients were included in infertile and fertile woman groups, respectively. Accordingly, the results cannot be extrapolated on the whole population and future studies on larger cohorts of women are of necessity.

Moreover, all women in our study were ethnically Caucasian, and thus the results cannot be generalized to women of other ethnicities. For example, Anahtar et al found differences between the vaginal microbiomes of asymptomatic young South African women and Caucasian women (9). Considering the mechanism of peristaltic uterine contractions, which contribute to the capture of different bacteria from the vagina into the uterus, it could be argued that the uterine microbiomes of women of different ethnic groups can differ extensively.

The absence of negative controls was another limitation of the study (10). Some of the detected microbes in our study are commonly found in the air and the soil and thus can be the causative agents of postoperative infections or infections of the airways (8).

The findings of this study could suggest that higher microbial diversity in women with infertility and burdened obstetric and gynecological anamnesis may be the reason for failed IVF attempts. In healthy women with no history of intrauterine manipulations or miscarriages, having a predominance of *Lactobacillus* of various types in the uterine microbiome can be a predisposing factor for favorable reproductive outcomes. It is well-established that *Lactobacillus* species are capable of inhibiting other bacteria by producing lactic acid and hydrogen peroxide (11). It is also known that the epithelium thickens and

Table 1. Relative Abundance of Microorganisms in the Studied Groups, Including Only Statistically Significant Differences

	1st Group (Infertile Women) n=22	2nd Group (Healthy Women) n=20	*P* Value
Lactobacillus iners	0.17 [0; 24.4]	37.2 [27.1; 44.6]	0.0005
Lactobacillus acidophilus	0 [0; 1.42]	7.24 [1.92; 12.6]	0.003
Lactobacillus jensenii	0 [0; 0]	5.1 [3.59; 7.95]	<0.0001
Lactobacillus crispatus	0 [0; 0]	7.38 [5.69; 10.2]	<0.0001
Methylobacterium aerolatum	0 [0; 0.21]	0 [0; 0]	0.01
Comamonas testosteroni	0 [0; 1.16]	0 [0; 0]	0.003
Gardnerella vaginalis	0 [0; 0]	0 [0; 0]	0.03*
Bifidobacterium gallinarum	0 [0; 0]	0 [0; 0]	0.03*
Prevotella melaninogenica	0 [0; 0]	0 [0; 0.05]	0.01
Bacteroides vulgatus	0 [0; 0]	0 [0; 0.05]	0.01
Corynebacterium bouchesdurhonense	0 [0; 0]	0 [0; 0.05]	0.02
Finegoldia magna	0 [0; 0.02]	0 [0; 0]	0.007
Bacteroides caccae	0 [0; 0]	0 [0; 0.005]	0.01
Bifidobacterium adolescentis	0 [0; 0]	0 [0; 0.005]	0.01

Note. Average values are presented as median (Lower quartile, Upper quartile). In addition, the comparison was carried out using the Mann-Whitney test.

* Symbol indicates the significance of differences at the zero median, and zero quartiles are explained by differences for the participants of the last quartile, which in particular, can be found by the different maximum concentrations presented in the following graphs (Figure 1).

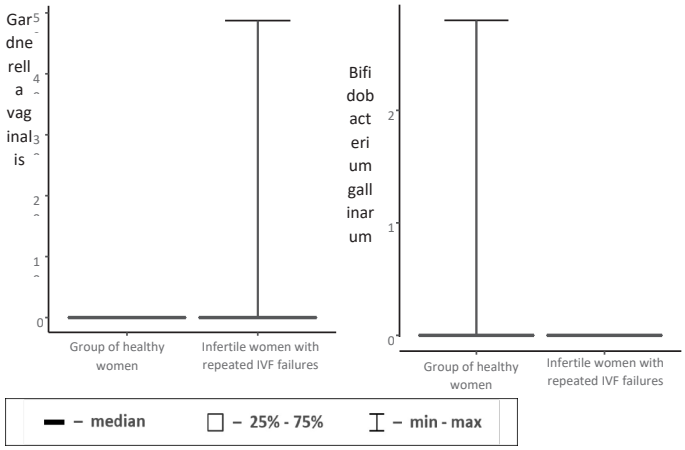

Figure 1. Boxplots of the Relative Abundance of *Gardnerella vaginalis* and *Bifidobacterium gallinarum* in the Endometrial Microbiota in the Two Groups.
Note. The bold line within the box is drawn to the median of each group, the bottom and top of the box to the 25th and 75th percentiles, respectively. Further, the whiskers are drawn to the 10th and 90th percentiles. Furthermore, boxplots show higher relative abundance of *Gardnerella vaginalis* in the group of infertile women while higher relative abundance of *Bifidobacterium gallinarum* in the group of healthy women.

releases glycogen under the influence of estrogens, thus favoring glucose-fermenting microorganisms such as *Lactobacillus* (12). Possibly, a thin endometrium, which is a rather common case in women with repeated IVF failures, is directly associated with the low abundance of *Lactobacillus* in the endometrial microbiome. Fang et al indirectly suggested this issue and reported that women with endometrial polyps had higher levels of *Lactobacillus* (13).

Moreover, this study included 3 women in the group with repeated IVF failures who had an abundance of *Lactobacillus* of more than 80% in the uterine microbiome. Therefore, further studies are needed regarding the relationship between the disturbed uterine microbiome and the markers of chronic endometritis (14).

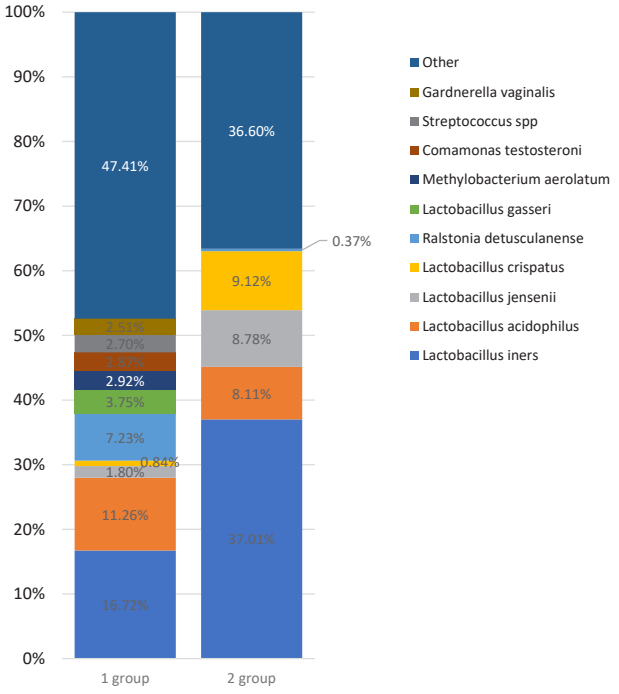

Figure 2. Relative Abundance of Most Common Species in the Endometrium of Women From the 1st Group (Infertile Women With Repeated IVF Failures) and the 2nd Group (Healthy Women With no Burdened Obstetric and Gynecological History).
Note. Bacterial composition is reported by color bars relative to a 100% scale. In addition, the results are presented by the taxonomic rank of species. "Other" species include different bacteria, in which relative abundance was less than 1%.

Conclusions

The findings of our study confirm the current understanding of the "lactobacillary" and "non-lactobacillary" microbiota of the endometrium and their effect on the success of embryo implantation. The concept of "normal" endometrial microbiota, as well as the analysis of dysbiotic microflora shifts in a close correlation with the presence of the histochemical markers of endometritis require further evaluations.

The question of the correction of endometrial dysbiosis remains open since the sequencing method, unlike the classical cultural method, provides no data about sensitivity to antibiotics. Currently, two methods of correctin g dysbiosis are generally used, including the administration of antibiotics, systemically or locally, and probio tics. Antibiotic resistance is generally a growing public health concern worldwide and represents a significant risk for the disruption of the normal microbiome. Therefore, the "blind" prescription of antibacterial drugs based only on sequencing results cannot be a favorable prospect for the treatment of endometrial dysbiosis.

Additionally, the administration of probiotics can be a promising and reasonable method for treating endometrial dysbiosis. Nowadays, two theories are generally accepted about the bacterial entry to the uterine cavity, including the ascending vaginal way and the hematogenous way from the oral cavity and intestines.

In experimental models on cows, Jeon et al showed that the same bacterial cultures were isolated from the blood and the uterine cavity (15). In another study, Fardini et al confirmed the hematogenous pathway by injecting human saliva into the tail veins of pregnant mice and later detecting microorganisms from the saliva in the placenta of the mice (3). Additionally, there are several publications confirming the presence of the same species of bacteria both in the human placenta and intestinal and oral cavities (16,17). The similarity of the intestinal, vaginal, and upper reproductive tract microbiomes gives a reason to hope for new strategies regarding treating genital dysbiosis with the transplantation of fecal microbiota as an alternative to aggressive antibiotic therapy (18).

Authors' Contribution

BVV wrote the paper, conceived and designed the analysis, collected data. SKM collected the data, performed the analysis. KNB conceived and designed the analysis. BIO conceived and designed the analysis. PDE performed the analysis, contributed analysis tool.AMV performed the analysis. DVV performed the analysis, contributed analysis tool.

Conflict of Interests

Authors declare that they have no conflict of interests.

Ethical Issues

The study was approved by the Local Ethics Clinical Research Committee of Rostov-on-Don State Medical University. Further, informed consent was obtained from all participants.

Financial Support

This study was financially supported by the Russian Science Foundation (grant № 19-75-00006) under the title of the "Role of the uterine microbiome in the genesis of reproductive losses and IVF failure.

References

1. Tissier H. Recherches sur la Flore Intestinale des Nourrissons (État Normal Et Pathologique). Paris: G Carré et C Naud; 1900.
2. Han YW, Redline RW, Li M, Yin L, Hill GB, McCormick TS. Fusobacterium nucleatum induces premature and term stillbirths in pregnant mice: implication of oral bacteria in preterm birth. Infect Immun. 2004;72(4):2272-2279. doi:10.1128/iai.72.4.2272-2279.2004
3. Fardini Y, Chung P, Dumm R, Joshi N, Han YW. Transmission of diverse oral bacteria to murine placenta: evidence for the oral microbiome as a potential source of intrauterine infection. Infect Immun. 2010;78(4):1789-1796. doi:10.1128/iai.01395-09
4. Perez PF, Doré J, Leclerc M, et al. Bacterial imprinting of the neonatal immune system: lessons from maternal cells? Pediatrics. 2007;119(3):e724-732. doi:10.1542/peds.2006-1649
5. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13(7):581-583. doi:10.1038/nmeth.3869
6. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007;73(16):5261-5267. doi:10.1128/aem.00062-07
7. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41(Database issue):D590-596. doi:10.1093/nar/gks1219
8. Khalki H, Deham H, Taghouti A, Yahyaoui G, Mahmoud M. [Comamonas testosteroni appendicitis]. Med Mal Infect. 2016;46(3):168-170. doi:10.1016/j.medmal.2015.12.009
9. Anahtar MN, Byrne EH, Doherty KE, et al. Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. Immunity. 2015;42(5):965-976. doi:10.1016/j.immuni.2015.04.019
10. Lauder AP, Roche AM, Sherrill-Mix S, et al. Comparison of placenta samples with contamination controls does not provide evidence for a distinct placenta microbiota. Microbiome. 2016;4(1):29. doi:10.1186/s40168-016-0172-3
11. Antonio MA, Hawes SE, Hillier SL. The identification of vaginal Lactobacillus species and the demographic and microbiologic characteristics of women colonized by these species. J Infect Dis. 1999;180(6):1950-1956. doi:10.1086/315109
12. Yamamoto T, Zhou X, Williams CJ, Hochwalt A, Forney LJ. Bacterial populations in the vaginas of healthy adolescent women. J Pediatr Adolesc Gynecol. 2009;22(1):11-18. doi:10.1016/j.jpag.2008.01.073
13. Fang RL, Chen LX, Shu WS, Yao SZ, Wang SW, Chen YQ. Barcoded sequencing reveals diverse intrauterine microbiomes in patients suffering with endometrial polyps. Am J Transl Res. 2016;8(3):1581-1592.
14. Liu Y, Ko EY, Wong KK, et al. Endometrial microbiota in infertile women with and without chronic endometritis as diagnosed using a quantitative and reference range-based method. Fertil Steril. 2019;112(4):707-717.e701. doi:10.1016/j.fertnstert.2019.05.015
15. Jeon SJ, Cunha F, Vieira-Neto A, et al. Blood as a route of transmission of uterine pathogens from the gut to the uterus in cows. Microbiome. 2017;5(1):109. doi:10.1186/s40168-017-0328-9
16. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J. The placenta harbors a unique microbiome. Sci Transl Med. 2014;6(237):237ra265. doi:10.1126/scitranslmed.3008599
17. Gomez-Arango LF, Barrett HL, McIntyre HD, Callaway LK, Morrison M, Nitert MD. Contributions of the maternal oral and gut microbiome to placental microbial colonization in overweight and obese pregnant women. Sci Rep. 2017;7(1):2860. doi:10.1038/s41598-017-03066-4
18. Quaranta G, Sanguinetti M, Masucci L. Fecal microbiota transplantation: a potential tool for treatment of human female reproductive tract diseases. Front Immunol. 2019;10:2653. doi:10.3389/fimmu.2019.02653