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Original Article

Lactobacilli Deficiency in Infertile Women Seeking IVF in Arash Hospital: An Imbalance in the Genital Microbiome

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Abstract

Objective: It is estimated that infertility affects approximately 9-30% of couples in their reproductive age and microorganisms may play an important role in such genital system dysfunction. The aim of this study was to investigate the presence of lactobacilli, *Gardnerella*, Enterobacteriaceae, and streptococci in the vagina, cervix and endometrium of women who referred for infertility and the healthy women who referred for oocyte donation.

Materials and methods: The endometrial, cervical and vaginal swab specimens were collected three days after the end of menstruation and cultured to isolate lactobacilli. DNA form these specimens was extracted and subjected to quantitative real-time PCR to determine the frequency of the above bacteria. All uterine biopsy samples were tested for the presence of bacterial DNA by PCR method.

Results: 94% of uterine biopsy samples contained bacterial DNA. The frequency of lactobacilli identified by real-time quantitative PCR in these two groups was 40% (endometrial samples), 70% (cervical samples), and 80% (vaginal samples), which differed from lactobacilli isolated by the culture method. The number of lactobacilli from cervical endometrium of healthy donors was higher than in the diseased group. There was a significant difference in the mean of *Gardnerella* bacteria in the cervix and endometrium and *Streptococcus* in the cervix ($p<0.05$).

Conclusion: Considering the decrease of lactobacilli and the increase of other bacteria, it is suggested to consider the composition and number of bacteria in the genital tract of asymptomatic infertile women as one of the possible causes of infertility.

Keywords: Infertility; Bacteria; *Gardnerella;* Enterobacteriaceae*;* Streptococci*; Lactobacillus;* Quantitative Real-Time PCR

Introduction

Infertility is defined as the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (1). Infertility is a multifactorial dysfunction but microbiological factors play an important role, as dysbiosis causes infertility by directly damaging the reproductive system or by

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affecting implantation and fertilization, as well as reducing sperm motility and viability (1, 2). Some studies suggest that the composition of the endometrial microbiota may affect implantation, pregnancy and live birth rates (2). Female reproductive microbiota may play a key role in the success of assisted reproductive technologies (3).

In general, the microbiota of the female genital tract is dominated by *Lactobacillus* species in a healthy state (3, 4). *Lactobacillus* dominated endometrial fluid and vaginal aspirate correlate with better outcomes (2). *Lactobacillus* eliminates other pathogens; when lactobacilli decrease other bacteria such as *Streptococcus* and *Gardnerella* can overgrow (4).

Actually, infertile women have a greater diversity of microorganisms and a decrease in the proportion of *Lactobacillus* in the vaginal microbiota (5). The non-*Lactobacillus* bacterial species that have been found to be associated with poor reproductive outcomes include *Streptococcus spp*, members of the Enterobacteriaceae family and *Gardnerella vaginalis* (6).

We used quantitative real-time PCR to investigate the possible role of bacteria in causing infertility and compared the frequency of *Lactobacillus*, *Gardnerella*, *Streptococcus* and members of the Enterobacteriaceae family in the uterus, cervix, and vagina of women referred for oocyte donation (control group) and women referred for oocyte and embryo freezing (25 women), women referred for myomas (n=11), and 4 women evaluated for polyps, ovarian cysts, and polycystic ovarian syndrome (case group). All samples were also cultured for lactobacilli.

Materials and methods

The present study was approved by the Ethics Committee of Tehran University of Medical Sciences. Written informed consent was obtained from all participants. 50 women aged 18 to 40 years referred to the in vitro fertilization (IVF) department of Arash Women's Hospital in Tehran were divided into two groups, donor egg and control group. Forty women who referred for infertility and 10 healthy women were candidates for egg or embryo freezing.

Exclusion criteria included use of hormonal contraception or IUD, use of antibiotics or probiotics in the past 8 weeks, abnormal Pap smear results in the past 3 years, vaginal bleeding and use of vaginal medications in the past 3 weeks, and sexual activity in the week prior to specimen collection. The treatment protocol for both groups was gonadotropin releasing hormone (GnRH) agonists.

Sample collection: Samples were collected three days after cessation of menses. Endometrial samples were collected by a gynecologist using a Pipelle (Medbar) under completely aseptic conditions. Cervical specimens were collected by speculum examination using Dacron swabs were gently rotated for 5 times to obtain cervical secretions. Vaginal mucosal samples were collected with a sterile swab.

Isolation of lactobacilli: *Lactobacillus* clones were isolated on Brain Heart Infusion and MRS agar plates. Both BHI and MRS agar plates were supplemented with 0.05% L-cysteine (7).

DNA extraction: DNA from endometrial samples was extracted using the GeneAll extraction kit (GeneAll Biotechnology/Korea) according to the instructions**.** Vaginal and cervical samples were centrifuged at 3500 × g for 5 minutes at 4°C, and bacterial DNA was extracted from the sediments. Bacterial DNA was stored at -20 °C.

Real-time quantitative PCR: To prepare an external standard, DNA was serially diluted in double-distilled water in the range of 10^6 to 10^3 copies according to the ABI's guidelines for "Generating standard curves with genomic DNA templates for use in quantitative PCR ". An aliquot of each dilution was stored at -20 °C until use (Table 1).

Table 1: Primers for PCR

Target	Primer	Sequence	Reference
Lactobacillus	F	5’-GAGGCAGCAGTAGGGAATCTTC-3’	(8)
	R	5’-GGCCAGTTACTACCTCTATCCTTCTTC-3’	
Enterobacteriaceae 16S rRNA	F	5’-CATTGACGTTACCCGCAGAAGAAGC-3’	(9)
	R	5’-CTCTACGAGACTCAAGCTTGC-3’	
Streptococcus spp.23S rRNA	F	5’-AGCTTAGAAGCAGCTATTCATTC-3’	(9)
	R	5’-GGATACACCTTTCGGTCTCTC-3’	
Gardnerella sp.	F	5′-GGGCGGGCTAGAGTGCA-3′	(10)
	R	5′-GAACCCGTGGAATGGGCC-3′	
Bacteria 16S rRNA	F	5’-GGGACCCGCACAAGCGGTGG-3’	(11)
	R	5’- GGGTTGCGCTCGTTGCGGGA -3’	

The amplification reaction was performed in a total volume of 20 µL, containing 3 µL of sample DNA, 0.8 µL of each primer (0.4 µmol/L), 10 µL of SYBR Green PCR Master Mix (2×), and 5.6 µL duplicate-distilled water. The amplification step was followed by a melting step at 94 °C for 1 min, followed by 40 cycles of denaturation at 94 °C for 10 s, annealing at (57 °C *Lactobacillus* spp, 57 °C *G. vaginalis* 30 s, 57 °C *Streptococcus*, 60 °C Enterobacteriaceae), extension at 72°C for 20 s, and a final extension at 72 °C for 5 min. Each qPCR assay was followed by this melting curve analysis, which allowed amplicon validation and false positives identification through its profile and the specific melting temperature. Experimental data were performed at least in three replicates and the results were expressed as mean ± SEM. A non-template control was included in each qPCR assay.

PCR for detection of 16S rRNA: All samples were checked with 16S rRNA primers after extraction. The PCR amplification mixture was 25 µL, with an initial denaturation step at 94°C for 5 minutes, followed by 40 cycles at 94 °C for 1 minute, 60 °C for 1 minute, and 72 °C for 1.5 minutes, and a final extension step at 72 °C for 10 minutes. Positive and negative bacterial controls were included in each analysis.

Statistical Analysis: Data were analyzed using SPSS software, version 26 (SPSS, Chicago, IL, USA). The Kolmogorov-Smirnov test was used to evaluate the normality of the distribution of the variables, and according to the above test and the significance levels of all the variables, which are reported as greater than 0.05, it can be concluded that the variables presented in the research follow a non-normal distribution, and in this research, Mann-Whitney U nonparametric test was used. Data were visualized using GraphPad Prism version 9 (GraphPad Software, Inc., San Diego, CA, USA).

Results

Isolation of vaginal lactobacilli: All samples were cultured on MRS containing L-cysteine and on BHI supplemented with L-cysteine, *Lactobacillus* was isolated from the endometrium (7.5%), cervix (10%) and vagina (42.5%) of the case group. *Lactobacillus* was isolated by culture from the endometrium (30%), cervix (20%), and vagina (80%) of the control group.

Standard Curves and Limit of Detection: Bacterial quantification standard curves were constructed from serial dilutions of genomic DNA extracted from each of the reference strains.

A highly linear correlation between the Ct values and the amount of each species (CFU/mL) was obtained for different standard curves with coefficients of determination (R2 values) ranging from 0.987 to 0.997 (Figure 1).

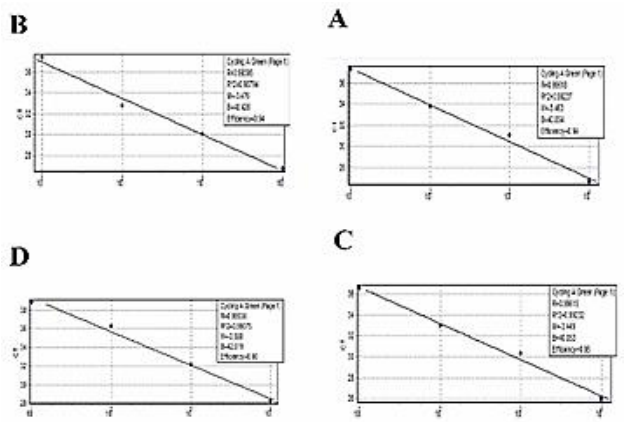

Figure 1: Representative standard curves obtained by plotting the average Ct values of lactobacilli (A), Gardnerella (B), Enterobacteriaceae (C) and streptococci (D)

In this study, 50 women were examined, 40 cases of infertile women of unknown cause (case group) and 10 healthy women were examined as the (control group). There was no evidenceof bacterial or viral infection in the genital tract in any of the control or infected individuals. We collected 3 samples from each person, including cervical and vaginal swabs and endometrial samples. A total of 150 samples were analyzed in this study, including 50 cervical samples, 50 vaginal samples, and 50 endometrial biopsy samples were analyzed. Bacterial counts were measured using the qPCR method and samples with less than 10 bacteria were considered negative. The frequency of *Lactobacillus* in endometrial, cervical and vaginal samples from egg donors compared to the case group was evaluated using qPCR. Using this technique, the frequencies of lactobacilli were 20% (endometrial samples), 50% (cervical samples) and 28 % percent (vaginal samples).

The average of vaginal *Lactobacillus* bacteria in the control group was higher than the case group, although this difference was not reported as a significant difference. Cervical and endometrial *Lactobacillus* have significant differences and the control group has a higher average than the case group ($p<0.05$).

There was no significant relationship between the mean Enterobacteriaceae in the vagina and endometrium of the two groups, but there was a significant relationship between the frequency of Enterobacteriaceae in the cervix of the two groups ($p < 0.05$).

There was no significant difference in the number of vaginal *Gardnerella* between the two groups, the case group has a higher average. The average of these bacteria in the cervix and endometrium was significantly different and the case group contained higher average number of this organism in average ($p < 0.05$).

The average of vaginal and endometrial streptococci between the two groups was not significantly different, the case group showed higher average. Cervical streptococci have a significant difference between the two groups ($p<0.05$).

In our study, the results of PCR for the detection of bacterial DNA were positive for all cervical and vaginal samples. In addition, 94% of uterine biopsy samples were positive for bacterial DNA in PCR.

Discussion

Infertility is estimated to affect approximately 9-30% of couples of reproductive age and is considered not only a private problem but also a public health burden. Several factors, including reproductive tract microorganisms, play a role in infertility. However, there are still no guidelines for the microbiological evaluation of infertile couples prior to in vitro fertilization (IVF)(12) . The purpose of this study was to investigate and compare the frequency of *Lactobacillus*, *Gardnerella* and *Streptococcus* and Enterobacteriaceae in the uterus, cervix and vagina of women referred for infertility and healthy women referred for oocyte donation using the Real-time quantitative PCR method. The culture method was also used to detect *Lactobacillus*. Differences were observed between the two groups. The mean values of *Lactobacillus* in the vagina, cervix and uterus were higher in the control group than in the case group. There was a significant relationship between the frequency of *Lactobacillus* in the cervix and endometrium in these two groups, which is consistent with previous studies. Studies have shown that a *Lactobacillus*-dominated uterus is associated with better pregnancy outcomes (13, 14). The results of the Bernabeu's study suggest that the vaginal microbiome may influence ART outcomes, as *Lactobacillus*-dominated profiles are associated with pregnancy success (15). In Zhao's study, infertile women showed significant changes in the vaginal microbiome, such as a decrease in the abundance of *Lactobacillus* (16). In Koedooder's study of women with a low percentage of *Lactobacillus* in their vaginal sample, they were less likely to have a successful embryo implantation (6). Differences in the composition and stability of the vaginal microbiota in pregnant and non-pregnant women suggest a relationship between these two factors (17).

Lactobacillus by producing lactic acid, bacteriocins, polysaccharides, peptidoglycans and hydrogen peroxide (H_2O_2) and lactate production can maintain the acidic and anaerobic environment of the vagina and protect it from bacterial pathogens (2, 18). In this study, we found a significant difference between the frequency of streptococci in the cervix and *Gardnerella* in the cervix and endometrium between case group and control groups: control group had a higher average. The average of the vagina and endometrium between the two groups has no significant difference in Enterobacteriaceae bacteria, control group have a higher average than healthy people, although this difference is not a significant difference reported.

Microbiological culture of the tip of the transfer catheter in patients undergoing IVF has shown that the presence of bacterial species in the uterine cavity at the time of embryo transfer has a negative effect on the rate of implantation and pregnancy. In fact, members of the Enterobacteriaceae family, *Streptococcus* spp and Gram-negative bacteria have been associated with decreased implantation rates and poor pregnancy outcomes (17). A significant presence of *Gardnerella* spp. does not favor achieving pregnancy (15). In a study investigating changes in the vaginal microbiota and associated metabolome in women with recurrent implantation failure, the abundance of *Lactobacillus* was negatively associated with the abundance of pathogens such as *Gardnerella* and *Streptococcus*, suggesting a protective function of *Lactobacillus* (18).

An increased prevalence of vaginal streptococci may be associated with lower rates of assisted reproductive technology (ART) success (19). Bacteria associated with poor reproductive outcomes include streptococci, members of the Enterobacteriaceae, and *G. vaginalis* (6).

The average difference in cervical variable in Enterobacteriaceae between case group and control

group showed a significant difference, and the case group have a higher average than control group, which is inconsistent with the previous results. The results of PCR for lactobacilli were confirmatory to previous studies.

Lactobacillus was isolated from the endometrium (7.5%), cervix (10%) and vagina (42.5%) of the control group and endometrium (30%), cervix (20%), and vagina (80%) of the case group. While the frequency of lactobacilli identified by real-time quantitative PCR method in these two groups was 40% (endometrial samples), 70% (cervical samples) and 80% (vaginal samples). The value of culture method in predicting the sterility of genital system is low, while PCR showed much higher positivity in this system. However, one of the limitations of the DNA-based techniques, including PCR and DNA sequencing methods is the lack of differentiation between live and dead bacteria (20-22).

In this study, 94% of uterine biopsy samples contained bacterial DNA, which is consistent with previous studies**.** In the study by Moreno and Mitchell, up to 95% of hysterectomy specimens contained bacterial DNA (17, 23). These results support the growing consensus that the endometrial cavity is not sterile (24).

It is also important to note that three endometrial biopsies (2 cases, and 1 symptomatic control) did not yield a PCR product for the 16S rRNA gene, and were therefore considered negative. This may suggest that some individuals do not harbor microbiota in their endometrium (24).

Microorganisms can affect the reproductive potential in different ways and to different degrees by causing complications such as adhesions, tubal mucosal damage, or tubal obstruction (25). Changes in the microbial composition activate the immune system and increase levels of pro-inflammatory cytokines, which can lead to an inflammatory response in the endometrium (2, 26). In addition, bacteria can induce various damages to spermatozoa through direct binding to spermatozoa or through toxins and metabolites secreted by bacteria, such as DNA fragmentation, acrosome disruption, and cell membrane peroxidation (27).

Recent studies have identified several bacterial species of bacteria as biomarkers of cervical and vaginal microflora to improve the accuracy of infertility diagnosis and treatment methods (13). Recently, it has been suggested that the composition of vaginal microbiota before IVF or IVF-ICSI treatment may predict pregnancy outcome (6).

Conclusion

According to the results of this study and its concordance with previous studies, it is suggested that the evaluation of the bacteria of the genital tract of asymptomatic infertile women should be considered as one of the methods to find the possible causes of infertility. Considering that 94% of uterine biopsy samples contained bacterial DNA, it can be concluded that the uterus is not sterile.

Conflict of Interests

Authors declare no conflict of interests.

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All procedures performed in studies involving human participants were in accordance with the ethics Committee of Tehran University of Medical Sciences (IR.TUMS.MEDICINE.REC.1400.427).

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