Bacterial infections of the lower genital tract in fertile and infertile women from the south-eastern Poland

Bakteryjne zakażenia dolnego odcinka dróg rodnych u płodnych i niepłodnych kobiet z terenu Polski południowo-wschodniej

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Abstract

Objectives: *The objective of the study was to investigate the detection rates of Chlamydia trachomatis, Neisseria gonorrhoeae, Mycoplasma genitalium, Mycoplasma hominis, Ureaplasma urealyticum, Gardnerella vaginalis, Escherichia coli, Streptococcus agalactiae and Enterococcus faecalis, showing no clinical signs of an ongoing, acute inflammatory state of the vagina and/or the cervix, in fertile and infertile women.*

Material and methods: *The study encompassed 161 women, including 101 women treated for infertility and 60 fertile women who had already given birth to healthy children. The material for the presence of C. trachomatis, N. gonorrhoeae, M. genitalium, M. hominis and U. urealyticum was collected from the cervical canal and analyzed by PCR. Furthermore, BD ProbeTec ET system was used to detect C. trachomatis infection. Vaginal swabs were collected for classification of bacterial vaginosis and aerobic vaginitis and assessed according to the Nugent score, as well as by traditional culture methods.*

Results: *U. urealyticum was identified in 9% of the infertile women and in 8% of controls. Presence of M. hominis was demonstrated only in the former (4%) and C. trachomatis only in latter (3%). N. gonorrhoeae and M. genitalium were not found in any of the examined women. The frequency of aerobic vaginitis in both groups was estimated at 12%. There were 7% bacterial vaginosis cases in the study group, and none in the control group (p=0.0096).*

Conclusions: *Despite having no symptoms of an ongoing acute inflammation of the reproductive tract, many women may experience permanent or periodic shifts of equilibrium of the vaginal and/or cervical microflora. BV develops more frequently in infertile patients when compared to the fertile women.*

Key words: **infertility** / **Chlamydia trachomatis** / **genital mycoplasmas** / / **bacterial vaginosis** / **aerobic vaginitis** /

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Streszczenie

Cel pracy: *Celem pracy było określenie częstości wykrywania Chlamydia trachomatis, Neisseria gonorrhoeae, Mycoplasma genitalium, Mycoplasma hominis, Ureaplasma urealyticum, Gardnerella vaginalis, Escherichia coli, Streptococcus agalactiae i Enterococcus faecalis u płodnych i niepłodnych kobiet, u których nie stwierdzono klinicznych parametrów toczącego się ostrego stanu zapalnego pochwy i/lub szyjki macicy.*

Materiały i metody: *Badaniem objęto w sumie 161 kobiet, w tym 101 kobiet leczonych z powodu niepłodności oraz 60 kobiet płodnych, które urodziły zdrowe dzieci. Materiał do badania w kierunku obecności C. trachomatis, N. gonorrhoeae, M. genitalium, M. hominis i U. urealyticum pobierany był z kanału szyjki macicy i analizowany metodą PCR. Ponadto do wykrywania zakażenia Chlamydia trachomatis wykorzystano system BD ProbeTec ET. Diagnostyka bakteryjnej waginozy (BV) i tlenowego zapalenia pochwy (AV) opierała się na ocenie materiału pobranego z tylnego sklepienia pochwy według kryteriów Nugenta oraz na tradycyjnych metodach hodowlanych.*

Wyniki: *Najczęściej izolowanym drobnoustrojem była U. urealyticum, którą zidentyfikowano u 9% kobiet niepłodnych i u 8% kobiet z grupy kontrolnej. Obecność M. hominis wykryto jedynie w grupie kobiet niepłodnych (4%), a C. trachomatis tylko w grupie kobiet stanowiących kontrolę (3%). U żadnej z przebadanych kobiet nie stwierdzono obecności takich drobnoustrojów jak N. gonorrhoeae i M. genitalium. Częstość występowania AV w obu porównywanych grupach oceniono na 12%. Rozpoznane przypadki BV stanowiły 7% w grupie kobiet niepłodnych, natomiast w grupie kontrolnej nie rozpoznano żadnego przypadku tego schorzenia (p=0,0096).*

Wnioski: *Pomimo braku klinicznych objawów toczącego się ostrego stanu zapalnego dróg rodnych, u wielu kobiet mogą występować przesunięcia równowagi biocenozy pochwy i/lub szyjki macicy. U kobiet niepłodnych znacznie częściej dochodzi do rozwoju BV w stosunku do kobiet płodnych.*

Słowa kluczowe: **niepłodność** / **Chlamydia trachomatis** / **mykoplazmy płciowe** / / **bakteryjna waginoza** / **tlenowe zapalenie pochwy** /

Introduction

Infertility has been estimated to concern approximately 13-15% of the world population. In Europe alone the problem affects several percent of couples trying for a baby, with 17.3% in the UK, 12.2% in France and 6.6% in Norway [1]. In Poland there have been no precise epidemiological studies on the topic but rough estimates say that from 700 thousand to a million couples seek treatment for infertility [2]. Owing to the systematic rise in the scale of the problem, the World Health Organization (WHO) has declared it a public condition and its treatment has since been considered as one of basic human rights [3].

Bacterial infections of the upper reproductive tract are often mentioned among the reasons for female infertility. Clinically, they manifest as pelvic inflammatory disease (PID) [4]. It is mainly sexually transmitted pathogens (*Chlamydia trachomatis, Neisseria gonorrhoeae, Mycoplasma genitalium, Mycoplasma hominis, Ureaplasma urealyticum*), as well as aerobic and anaerobic bacteria present in vaginal microflora, that are responsible for the development of PID [5].

Until recently, bacterial vaginosis (BV), the condition where *Gardnerella vaginalis* is one of the most commonly isolated agents, was considered to be only a quantitative disturbance of the bacterial microflora composition, with no further medical consequences.

Currently, it is known that untreated BV may lead to many complications. In pregnant women bacteria that are responsible for BV may cause infections of the decidua, chorion and amniotic fluid, which may result in premature rupture of the membranes, preterm delivery, low birth weight of the infant and also perinatal newborn and mother infections [6-8]. Untreated BV may also have a negative influence on female fertility. Epidemiological studies on women qualified to in vitro procedures revealed that patients with fallopian tube obstruction and no ovulation had a three-fold higher incidence of BV and PID than women with other reasons for infertility [9].

When discussing bacterial infections of the female genital tract, it is worthwhile to mention the condition known as aerobic vaginitis (AV), an inflammation of the vagina with uncontrolled overgrowth of aerobic bacteria, like *Escherichia coli, Streptococcus agalactiae* or *Enterococcus faecalis* over *Lactobacillus spp.* AV commonly results from improper treatment of BV, vulvo-vaginal candidiasis (VVC) and trichomoniasis [10]. To the best of our knowledge, literature lacks reports which show a direct link between AV and infertility, but similarly to BV, this condition may also contribute to other infectious complications which may lead to infertility. Awareness of these risk factors has resulted in the introduction of BV/AV screening programs in many countries.

Objective

The aim of the work was to assess the incidence of bacterial etiological factors causing inflammation of the upper and lower reproductive tract in woman treated for infertility with no clinical parameters of acute inflammation of the vagina and/or the cervix.

Material and Methods

161 women, aged from 20 and 40 years, from South-Eastern Poland were included into the study. The first group (N=101) was composed of woman treated for infertility for over one year at the PARENS centre for the treatment of infertility in Cracow. The study was supported by grant no 2PO5E 004 30. Women and their partners had been thoroughly investigated to exclude other factors which may have played a role in problems with conception, such as anatomical and hormonal abnormalities, endometriosis and abnormal sperm parameters. At the time the samples were collected for microbiological studies, none of the

patients complained of symptoms from their reproductive tract. The remaining 60 women, with no history of fertility problems and at least one child, comprised the control group. Similarly to the women from the study group, the controls did not present with any clinical symptoms of inflammation of the reproductive tract.

All study participants signed an informed consent. Material samples were collected at the Outpatient Clinic of Diagnostics, Department of Microbiology, Jagiellonian University Medical College, based on the referral form from the investigating gynecologist. Women receiving antibiotic therapy or up to three weeks after the treatment were excluded from the study. The material was obtained from the posterior vaginal fornix and the cervical canal (swabs), as well as urine (first-catch urine specimens containing epithelial cells).

Microbiological studies were performed to detect typical etiological agents causing infections of the lower and upper reproductive tract, i.e.: *C. trachomatis, N. gonorrhoeae, M. genitalium, M. hominis, U. urealyticum*, *G. vaginalis, E. coli, S. agalactiae, E. faecalis.*

The material from the cervical canal was collected and tested for the presence of *C. trachomatis, N. gonorrhoeae, M. hominis, M. genitalium* and *U. urealyticum* based on PCR according to the procedure described in the literature [11-14]. PCR was performed using species-specific primers, with their sequences and amplicon size listed in Table I. Columnar DNA Genomic Mini set (DNA Gdańsk, Poland) was used for the isolation of the genetic material.

Additionally, 66 patients (31 women with infertility and 35 controls) were randomly selected to have urine samples collected. Urine samples were tested using Strand Displacement Amplification (SDA) technology with BD ProbeTec ET (Becton Dickinson, USA) apparatus to detect *C. trachomatis* DNA [15]. The collected samples were processed and analyzed according to the producer's recommendations and methodology.

The material collected from the posterior vaginal fornix was examined for the presence of bacterial (both aerobic and anaerobic) as well as fungal agents of vaginal infections. pH measurement was an additional parameter facilitating the determination of the status of physiological vaginal microflora, performed using a colorimetric pH PEHANON indicator test (Macherey-Nagel, Germany), which allowed to determine pH on a scale from 3.8 to 5.5. Two swabs were collected from each woman. One of them was used to prepare a Gram-stained specimen for direct evaluation of the vaginal microflora according to the 10-point Nugent score [16], and the second swab was used for qualitative and quantitative cultures for bacterial and fungal agents of vaginal infections.

The material was placed in 1 ml of liquid Schaedler medium (Difco, USA), mixed and serially diluted 4 times and then streaked on solid differentiating media. Aerobic bacteria were cultured on Columbia Agar with 5% sheep blood (Difco) incubated in aerobic conditions for 24h at 37°C. The same culture conditions were applied to *Enterobacteriaceae* cultured on McConkey's agar (Biocorp, Poland) and enterococci cultured on Bile Esculin Azide LAB-agar (Biocorp). Schaedler medium (Difco) with vitamin K and 5% sheep blood was used for strictly anaerobic bacteria. Cultures of *G. vaginalis* were made on Columbia Blood Agar Base (Difco) with 10% human blood and Selective Supplement (Oxoid, UK), whereas MRS Agar (Oxoid) was used for lactobacilli. Anaerobic bacteria were cultured in strictly anaerobic conditions in MACS (Don Whitley, UK) anaerobic chamber, at 37°C for 48h. Streaked Sabouraud Agar (Difco) was incubated in aerobic conditions for 24h at 37°C to detect yeast-like fungi. Cultured bacterial and fungal colonies were counted to determine colony forming units (c.f.u.) per 1 ml of the medium in which examined sample was suspended. Microorganisms not belonging to the *Lactobacillus* genus, the population of which was equal to or smaller than 1.0×10^4 c.f.u/ml, were considered normal bacterial flora of the vagina. Each bacterial or fungal etiological factor with the population equal to or greater than 1.0×10^5 c.f.u/ml, was considered to be disturbing the vaginal ecosystem equilibrium. For precise identification of the species in the cultured colonies the following API (bioMérieux, France) tests were used: API STREP (for streptococci and enterococci), API STAPH (for staphylococci), API 20E (for *Enterobacteriaceae*) and API 20A (for anaerobes). The results were analyzed with API LAB software used for classifying studied bacteria.

G^2 (Likelihood Ratio) test was used for statistical analysis. Level of significance was established at $p<0.05$. Statistical analysis was made using the JMP 7.0.2 (SAS, USA) software package.

Results

The analyses of the material collected from the cervical canal confirmed that in both groups of women *U. urealyticum* was the most commonly detected pathogen. It was found in 9 women (9%) treated for infertility and in 5 women (8%) from the control group. No statistically significant difference was shown between the two groups for the occurrence of this etiological factor (p=0.9248).

M. hominis was the second most commonly detected microorganism. It was found in 4 women (4%) treated for infertility but was not detected in controls. The difference was on the verge of statistical significance (p=0.0515). It is worth noticing that 3 of 4 women with *M. hominis* were *U. urealyticum* positive.

The presence of *C. trachomatis* in the cervical canal was confirmed in two women (3%) from the control group. No infection with this pathogen was found in the study group. The difference proved to be statistically significant (p=0.0458).

Apart from the conventional PCR method for detecting *C. trachomatis* infections, the SDA based on the BD ProbeTec ET genetic system was used as comparison. From the 66 examined urinary samples, SDA showed a negative result in 64 cases (97%), 1 doubtful result (1.5%) and 1 positive result (1.5%). The positive result obtained with SDA was also confirmed using PCR with species-specific primers. The second positive result for *C. trachomatis* shown by PCR was impossible to confirm with BD ProbeTec ET owing to non-validated urine sample. PCR examination of the doubtful SDA result turned out to be negative.

None of the women tested positive for *N. gonorrhoeae* or *M. genitalium.* The results are shown in Table II. Additionally, Fig. 1 demonstrates an example of PCR reaction products for *U. urealyticum,* performed with species-specific primers.

In both groups, the majority of woman had normal bacterial vaginal flora. Normal bacterial vaginal flora was confirmed in 80 women (79%) treated for infertility and 51 women (85%) from the control group.

Table I. Nucleotide sequences of primers and the reference strains of bacteria.

Pathogen	The primer sequences 5'→ 3'	Amplicon size	Reference strains
Chlamydia trachomatis	**NLO** ATG AAA AAA CTC TTG AAA TCG **NRO** CTC AAC TGT AAC TGC GTA TTT	1100 pz	DSMZ 19102
Neisseria gonorrhoeae	**HO1** GCT ACG CAT ACC CGC GTT GC **HO3** CGA AGA CCT TCG AGC AGA CA	390 pz	ATCC 19424
Mycoplasma genitalium	**MG1** AGT TGA TGA AAC CTT AAC CCC TTG G **MG2** CCG TTG AGG GGT TTT CCA TTT TTG C	282 pz	DSMZ 19775
Mycoplasma hominis	**RNAH1** CAA TGG CTA ATG CCG GAT ACG C **RNAH2** GGT ACC GTC AGT CTG CAA T	334 pz	DSMZ 19104
Ureaplasma urealyticum	**UMS125** GTA TTT GCA ATC TTT ATA TGT TTT CG **UMA226** CAG CTG ATG TAA GTG CAG CAT TAA ATT C	448 pz	DSMZ 21275

Table II. The number and percentage of specimens positive for C. trachomatis, N. gonorrhoeae and genital mycoplasmas with the PCR assay.

Causative agents of PID	Group of infertile women (N = 101)		Group of fertile women (N = 60)	
	Number of cases	%	Number of cases	%
Chlamydia trachomatis	0	-	2	3
Neisseria gonorrhoeae	0	-	0	-
Mycoplasma hominis	4	4	0	-
Mycoplasma genitalium	0	-	0	-
Ureaplasma urealyticum	9	9	5	8

BV was confirmed (based on pH, Nugent score and quantitative culture results) in 7 women (7%) treated for infertility, and none from the control group. Statistical analysis showed that infertile women had significantly more frequently developed BV in comparison to fertile women (p=0.0096).

AV was diagnosed in 12 women (12%) treated for infertility and 7 controls (12%). No statistically significant difference was found (p=0.9674). Furthermore, 2 women (2%) from the study group and 2 women (3%) from the control group were diagnosed with VVC. Results showing the vaginal microflora of women participating in the study are listed in Table 3, and Fig. 2 was used to illustrate the frequency of BV, AV and VVC in both groups.

Despite the fact that no acute clinical symptoms of an ongoing inflammation of the vagina and/or cervix were found in women from the two examined groups, the presence of pathogenic microorganisms was detected in 29/101 infertile women (28.7%) and in 16/60 controls (26.6%).

Discussion

The results of microbiological examinations of materials collected from the cervical canal of fertile and infertile women are similar to results reported by other authors. In a study based on the PCR technique, including 31 infertile women and 31 controls, no differences as to the frequency of *C. trachomatis, U. urealyticum* and *M. hominis* were found and the most commonly isolated pathogen was *U. urealyticum* [17]. In a recently published study, Günyeli et al., demonstrated that there were no significant differences as to the detection rates of infections with *C. trachomatis, M. hominis* and *U. urealyticum* in fertile and infertile women and men. The study was performed on a group of 212 participants, using ELISA [18]. In Poland, a study performed by Grześko et al., evaluated the detection rate of *U. urealyticum* and *M. hominis* from the cervical canal of fertile and infertile women. The results of the study showed that *U. urealyticum* was present with high frequency in the material collected from the cervical canal in both groups, but without significant differences.

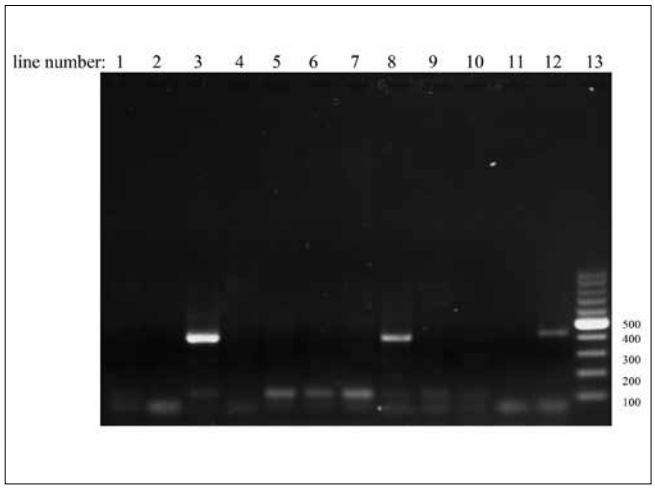

Figure 1. Figure 1. The result of PCR assay that detected *U. Urealyticum.* Lanes 1-10 samples from patients, lane 11 negative control, lane 12 positive control (*U. urealyticum* DSMZ 21275), lane 13 size marker (Perfect 100bp DNA Ladder, EURx). A positive PCR result is indicated by the presence of a 448 bp product.

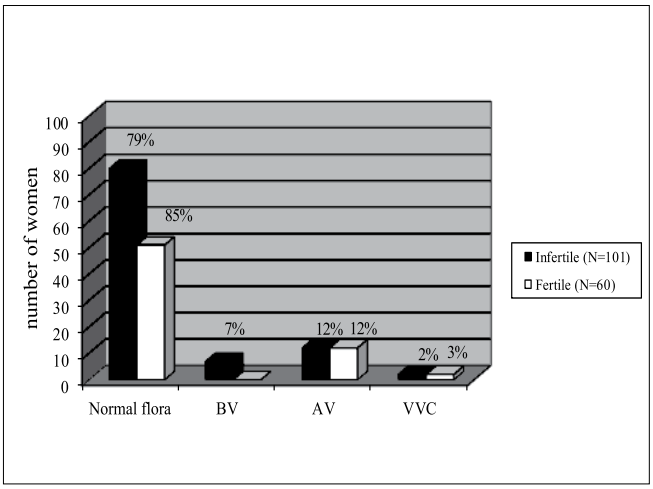

Figure 2. The frequency of bacterial vaginosis (BV), aerobic vaginitis (AV) and vulvo-vaginal candidiasis (VVC) among fertile and infertile women.

Table III. The assessment of the lower genital tract microflora among study participants. BV (bacterial vaginosis), AV (aerobic vaginitis), VVC (vulvo-vaginal candidiasis), N/A (not applicable).

parameters	INFERTILE WOMEN				FERTILE WOMEN			
	Normal flora	BV	AV	VVC	Normal flora	BV	AV	VVC
number of women	80	7	12	2	51	0	7	2
average pH	4,6 ± 0,4	5,3 ± 0,2	5,0 ± 0,4	4,6 ± 0,4	4,7 ± 0,4	-	5,2 ± 0,5	4,5 ± 0,2
Number of pathogenic bacteria	**≤1,0x10^4 cfu/ml**	**≥1,0x10^5 cfu/ml**	**≥1,0x10^5 cfu/ml**	-	**≤1,0x10^4 cfu/ml**	**≥1,0x10^5 cfu/ml**	**≥1,0x10^5 cfu/ml**	-
Gardnerella vaginalis	-	7	-	N/A	1	-	-	N/A
Escherichia coli	8	-	3	N/A	1	-	1	N/A
Streptococcus agalactiae	-	-	6	N/A	3	-	2	N/A
Enterococcus faecalis	14	-	3	N/A	10	-	4	N/A

The presence of this microorganism was found in 72% of the infertile women and in 82% controls. *M. hominis* was present in both groups, also with no significant differences. Its presence was detected in 5.9% of the infertile women and 8.7% of the women from the control group [19]. In a study of Al-Ramahi et al., the authors, using PCR, stated that there were no significant differences in the isolation rates of *C. trachomatis* from the cervical canal between the groups of infertile and fertile women, even though this pathogen was isolated relatively more frequently from infertile women when compared to controls (3.9% and 0.7%, respectively) [20].

The results of different studies discussed above and the results of our study show that bacterial infections of the lower and upper reproductive tract of fertile women and women having problems with natural conception occur at similar rates. Obviously, since the association of *C. trachomatis* and *N. gonorrhoeae* with infertility has been proven, early detection and treatment is of the utmost importance, especially in the higher risk groups, e.g. sexually active adolescents [21].

It is especially important to prevent pelvic inflammatory conditions that may lead not only to infertility but also to pregnancy loss [4].

The exact role that infections with genital mycoplasmas play in infertility remains unclear. Although there are numerous reports which point to the fact that these bacteria may in fact be the reason for problems with fertility [22,23,24], there are also many publications which argue otherwise [18,19,25]. It is a known fact that in pregnant women infections with these microorganisms may be the cause for recurrent miscarriages, preterm birth and low birth weight of the infants [26,27,28,29]. It is also worth noting that despite *C. trachomatis* being still the most common sexually transmitted pathogen, the percentage of women infected with genital mycoplasmas (and also especially *U. urealyticum*) has increased recently, what is confirmed by our study [18,19,30].

Review of relevant literature reveals that sexually transmitted microorganisms are significantly more common in infertile women in comparison to controls. Badami et al., demonstrated that in 125 infertile and 250 fertile women the infection rates were, respectively, 8.8% and 0.8% for *C. trachomatis*, 35.6% and 7.2% for *M. hominis*, and 32.8% and 19.2% for *U. urealyticum*. The differences between the groups were statistically significant. The evaluation was based on indirect immunofluorescence test and standard culture method [31]. In a recent paper, Zhou et al., examined 327 women with confirmed Fallopian tube infertility and 286 healthy, pregnant controls. The estimated rates of *C. trachomatis* infection were on average 15% in the infertile women and 2.8% in the control group; 23% and 7% for *U. urealyticum* and 29% and 8% for *M. hominis*, respectively [32].

The considerable divergence of the results is owed to the fact that the epidemiological analyses take place in different parts of the world and different diagnostic methods are used. In our study (classic PCR and SDA based on BD ProbeTec ET set), we used assays which allowed to detect even a single DNA of the sought microorganisms and both study methods gave comparable results. Also, we did not use diagnostic culture method owing to fastidious growth demands of the microbes and prolonged waiting time. Similarly, serological methods were not employed, since they have little value for diagnosing asymptomatic infections, with lower number of microorganisms [33].

The obtained results show that among women experiencing problems with conception, BV develops significantly more often when compared to fertile women. Data from the medical literature suggest that disturbances of bacterial vaginal microflora composition, like BV, are related to increased levels of proinflammatory cytokines, such as IL-8, IL-1β, interferon gamma (INF-γ) or tumor necrosis factor-alpha (TNF-α), which impair the activity of the immune system related to mucous membranes, and may be the reason for the so-called idiopathic infertility [34,35]. Results of the studies performed by Mania-Pramanik et al., also detect a statistically significant relationship between BV and infertility. Among 510 infertile women in that study, 14.1% were diagnosed with BV [36]. In case of AV, results of our study did not find any difference regarding its incidence in the group of fertile women vs. women from the control group. So far, there have been no reports in the literature showing a relationship of AV and infertility, but some studies report that, similarly to BV, it may be a reason for premature delivery and perinatal maternal and neonatal infections [10,37,38]. Bearing in mind those complications, it is worthwhile to investigate this disease entity more closely, especially since it affects a significant number of women worldwide.

Conclusion

1. The absence of clinical symptoms of an ongoing acute inflammatory state in the genital tract does not rule out the possibility of such etiological agents causing inflammation being present in small populations and keeping the inflammatory process of the genital tract around the subthreshold level.
2. Quantitative and qualitative vaginal microflora studies of infertile women have shown that imbalance of the vaginal ecosystem and the development of BV affect them more frequently than fertile women.

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