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INFECTIOUS DIAGNOSTICS ON MENSTRUAL TISSUE IN REPRODUCTIVE PROBLEMS

ABSTRACT

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2. Assoc. Prof. Maria Dimitrova Dragneva, PhD (opinion)
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The materials for the defence are available at the Department of Medical Chemistry and Biochemistry, Medical University - Sofia.

ABBREVIATIONS USED:

CMV - Cytomegalovirus

EBV - Epstein-Barr virus

HHV - Human Herpes viruses

HHV-6 - Human herpesvirus 6

HHV-7 - Human herpesvirus 7

HHV-8 - Human herpesvirus 8

HSV - Herpes simplex virus

HSV1 - Herpes simplex virus type 1

HSV2 - Herpes simplex virus type 2

IVF - In vitro fertilisation

NK - Natural killer cells

PCR - Polymerase chain reaction

VZV - Varicella-zoster virus

DNA - Deoxyribonucleic acid

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INTRODUCTION

Infertility affects nearly 10% of the global population of reproductive age, with 200,000 couples in Bulgaria being diagnosed with it, making it a national medical and social problem.

The current dissertation focuses on the infectious etiology of female infertility and proposes a new approach for non-invasive diagnosis of upper genital tract endometrial infections using a biological sample - menstrual tissue. Menstrual tissue contains parts of the functional layer of the endometrium (decidua) and encompasses the specific perimeter of viral/bacterial activity and future embryo implantation.

In this dissertation, molecular infectious disease analyses for a bacterial-viral panel (Chlamydia trachomatis, Ureaplasma urealyticum/ parvum, Mycomplasma hominis/genitalium, Gardnerella vaginalis, HSV1/2, EBV, CMV, VZV, HHV-6, HHV-7, HHV-8) were conducted on 335 females of Bulgarian origin, divided into the following groups: Clinical infertile group (180 females with a biological sample of menstrual tissue and diverse medical history); Control infertile group (65 females withs biological sample of endometrial biopsy and history of miscarriage); Healthy control group (90 fertile females with a biological sample of menstrual tissue).

The infertile control group, represented by females with endometrial biopsy samples, served for comparative analysis with the clinical infertile group to demonstrate the feasibility of using menstrual tissue as a non-invasive biological sample, equivalent in representativeness to invasive endometrial biopsy.

In the infertile clinical group, positive infections were detected in 61.1% (48.8% bacterial and 22.2% viral), corresponding to the data in the clinical control group: 64.53% (49.23% bacterial and 15.3% viral pathogens). The healthy control group studied turned out to be 100% negative regarding the target diagnostic panel. *Gardnerella vaginalis* and *Ureaplasma parvum* bacteria were detected in 69.31% and 61.36% of all positive menstrual tissues and 31.25% and 3.12% of all bacterially infected endometrial biopsies, respectively. Active infection with *Mycomplasma hominis* and *Ureaplasma urealyticum* was detected at an equivalent frequency of 2.27% in menstrual tissues positive for bacterial pathogens. Active infection with *EBV, CMV, HHV-6, HHV-7* was found in 30%, 30%, 20% and 20% of viral-factor positive endometrial biopsies and in 40%, 7.5%, 10% and 42.5% of menstrual tissues, respectively. The following target pathogens were not detected in any of the menstrual tissues of the probands studied: *Chlamydia trachomatis, Mycomplasma genitalium, HSV1, VZV, and HHV-8*. In the endometrial biopsies, the following were not detected*: Chlamydia trachomatis, Ureaplasma urealyticum, Mycoplasma hominis/genitalium, HSV1/2, VZV, and HHV-8.*

In addition to the targeted bacterial-viral panel, endometrial biopsies were also tested for endometrial microbiome status, including aerobic, anaerobic, opportunistic bacteria, and commensals. Anaerobic and aerobic dysbacteriosis was detected in 53.33% and 27% in all bacterially infected endometrial samples. A similar study on menstrual tissue probands is not present due to inability of applying the method used on this type of sample.

The observed similar rates of positive infections in the infertile group examined using menstrual tissue (61.1%), and the control infertile group, examined using endometrial biopsy sample (64.53%), demonstrate the representativeness of non-invasive menstrual tissue as a sample of the upper female genital tract. They also affirm the feasibility of using it as a substitute for invasive endometrial biopsy. In both groups, an absolute agreement was observed in the percentage of patients with positive infectious status and their distribution based on bacterial or viral etiology. At the species-specific level, menstrual tissue and endometrial biopsy

exhibited certain percentage differences in the pathogens detected, attributable to the sample heterogeneity and the patients' medical histories.

The reported negative infectious status in 100% of the investigated healthy controls clearly underscores the significance and adverse impact of the target infectious panel on female infertility.

Further evidence of the representativeness of menstrual tissue is the fact that, during parallel testing of patient samples (15% of the control infertile group) with both types of samples - endometrial biopsy and menstrual tissue - we achieved 100% concordance of results.

The primary focus of this current study, namely delineating a target viral-bacterial panel in infertility and developing a new approach for non-invasive diagnosis of upper female genital tract infections through menstrual tissue analysis, contributes to streamlining diagnostic practices and elucidating the infectious etiology behind reproductive failures. This is of paramount importance for implementing a new screening diagnostic algorithm in women experiencing reproductive difficulties and administering tailored individualized therapy.

The achieved natural concept, following therapy in 97% of cases with endometrial dysbiosis and a positive infectious status regarding the target range, serves as unequivocal evidence of the benefit derived from the application of the introduced diagnostic and monitoring algorithm.

OBJECTIVE AND TASKS

OBJECTIVE

To investigate the role of bacterial and viral pathogens (Chlamydia trachomatis, Ureaplasma urealyticum/ parvum, Mycomplasma hominis/genitalium, Gardnerella vaginalis, HSV1/2, EBV, CMV, VZV, HHV-6, HHV-7, HHV-8) in females with reproductive failures and to implement a non-invasive diagnostic model for their detection in the upper genital tract using biological material - menstrual tissue.

TASKS

- Selection of clinical groups and compilation of patient records of study participants containing detailed medical information associated with infertility.
- Optimization of a molecular biology methodology for isolating total DNA from biological samples: menstrual tissue and endometrial biopsy.
- Implementation of a molecular-biological method for amplifying *Chlamydia trachomatis* and *CD* housekeeping gene.
- Determination of the overall frequency of infection with the target viral-bacterial panel in the clinical groups.
- Assessment of clinicopathological status and species-specific infection/co-infection rates with the study target *microorganisms (Chlamydia trachomatis, Ureaplasma urealyticum/ parvum, Mycomplasma hominis/genitalium, Gardnerella vaginalis, HSV1/2, EBV, CMV, VZV, HHV-6, HHV-7, HHV-8)* in menstrual tissue from infertile women (clinical infertile group).
- Determination of species-specific frequency of infection/co-infection with the microorganisms targeted in the study *(Chlamydia trachomatis, Ureaplasma urealyticum/parvum, Mycoplasma hominis/genitalium, Gardnerella vaginalis, HSV1/2, EBV, CMV, VZV, HHV-6, HHV-7, HHV-8)* in endometrial biopsy samples from infertile women (control infertile group).
- Determination of endometrial microbiome status in endometrial biopsies from infertile females (infertile control group).
- Comparative analysis of clinical and control infertile group data regarding the application of the target viral-bacterial panel on menstrual tissue and endometrial biopsy specimens.
- Summarization and interpretation of the clinical and molecular infectious results of the present study.
- Establishment of a biobank containing isolated total DNA arrays from menstrual tissue and endometrial biopsies of female patients with reproductive failures of Bulgarian origin.

MATERIALS AND METHODS

MATERIALS

1. BIOLOGICAL MATERIAL

In this dissertation, analyses were performed on two types of biological material: menstrual tissue and endometrial biopsy. The biological material served as a selection criterion for categorizing the clinical groups.

2. CLINICAL MATERIAL

The molecular-infectious studies were conducted on 335 females of Bulgarian origin divided into the following clinical groups:

Infertile group - 180 female patients with a history of infertility including: miscarriage or recurrent ones, difficulties in natural conception, physiological obstruction of anatomical compartments of the female genital tract due to inflammation or surgical interventions, unsuccessful assisted reproduction, autoimmune or other concomitant diseases. The analyses were performed on a biological sample- menstrual tissue.

Infertile control group - 65 patients with a history of infertility, including solely a history of miscarriage or recurrent ones, and absence of the health problems associated with infertility as described in the previous group. Analyses were performed on a biological sample - endometrial biopsy, with the aim of comparing the non-invasive menstrual tissue with the invasive biopsy.

Healthy control group - 90 healthy fertile female patients. Analyses were performed on a biological sample - menstrual tissue.

The probands from all groups were analyzed and selected with negative cervicovaginal infection status with respect to the target diagnostic panel. The purpose of this selection criterion was to eliminate the risk of cervicovaginal contamination of endometrial samples (menstrual tissue and endometrial biopsy) and to ensure analytical precision solely within the upper genital tract.

All infertile and fertile females included in the study were aged 22-49 years and of Bulgarian origin.

The probands were clinically diagnosed with infertility according to established standards and referred for DNA analysis by medical collaborators - obstetricians and assisted reproduction specialists.

METHODS

1. PRE-ANALYTICAL PROCESSING OF THE MATERIAL

1.1 Collection and pre-analytical processing of biological samples: menstrual tissue and endometrial biopsy.

2. ANALYTICAL PROCESSING OF THE MATERIAL

2.1. Isolation of total DNA from pre-processed menstrual tissue/endometrial biopsy.
2.2. Polymerase Chain Reaction (PCR).

- PCR amplification of a targeted region of the *Chlamydia trachomatis* genome and *CD* housekeeping gene.

2.3. Agarose gel electrophoresis

2.4. Real time PCR – a method for qualitative/quantitative analysis

- Real time PCR amplification of bacterial pathogens:

- *Ureaplasma urealyticum*
- *Ureaplasma parvum*
- *Mycomplasma hominis*
- *Mycomplasma genitalium*
- *Gardnerella vaginalis*

- Real time PCR amplification of viral pathogens:

- *Herpes simplex virus type 1 (HSV1)*
- *Herpes simplex virus type 2 (HSV 2)*
- *Epstein-Barr virus (EBV)*
- *Cytomegalovirus (CMV)*
- *Varicella-zoster virus (VZV)*
- *Human herpesvirus 6 (HHV-6)*
- *Human herpesvirus 7 (HHV-7)*
- *Human herpesvirus 8 (HHV-8)*

- Real time PCR amplification of endometrial microbiome:

Normal microflora:

- *Lactobacillus spp.,*

Facultative anaerobic microorganisms:

- *Enterobacterium spp.,*
- *Streptococcus spp.,*
- *Staphylococcus spp.,*

Obligate anaerobic microorganisms:

- *Gardnerella vaginalis., Prevotella bivia., Porphyromonas spp.,*
- *Eubacterium spp.,*
- *Sneathia spp., Leptotrichia spp., Fusobacterium spp.,*
- *Megasphaera spp., Veillonella spp., Dialister spp.,*
- *Lachnobacterium spp., Clostridium spp.,*
- *Mobiluncus spp., Corynebacterium spp.,*
- *Peptostreptococcus spp.,*
- *Atopobium vaginae.,*

Mycoplasmas:

- *Ureaplasma urealyticum, Ureaplasma parvum.,*
- *Mycoplasma hominis,*
- *Mycoplasma genitalium.,*

Mycoses:

Candida spp.,

RESULTS AND DISCUSSION

1. Selection of clinical groups and compilation of patient records of study participants containing detailed medical information associated with infertility.

Clinical groups

The subject of molecular infectious studies in the present dissertation were 335 females of Bulgarian origin aged 22-49 years, divided into three clinical groups based on the type of biological material and history of reproductive failure, including specific pathologies and medical information associated with infertility (Table 1).

Table 1. Clinical groups participating in the study.

Clinical group	**Diagnosis:**	**Anamnesis:**	**Number of patients:**	**Biological sample:**
Infertile	**Infertility**	• Recurrent miscarriages • Difficulties with natural conception • Physiological obstruction of anatomical compartments of the female genital tract, /result of inflammation or surgical interventions/ • Unsuccessful IVF procedures • Presence of autoimmune disease and/or other concomitant diseases, etc.	**180**	**Menstrual Tissue**
Infertility control group	**Infertility**	• History of miscarriage or recurrent miscarriage only • Absence of any additional health problems associated with reproductive failure.	**65**	**Endometrial biopsy**
Healthy control group	No issues with infertility	• N/A	**90**	**Menstrual Tissue**

The selection of menstrual tissue as the target biological sample is optimal choice due to the fact that classical biological samples, informative about the state of the endometrium (endometrial biopsies), are invasive. Endometrial biopsies are not a routine option, especially for screening assessment of upper genital tract infection status. Hence, in order to minimize the possibility of hidden undiagnosed endometrial infections, menstrual tissue was selected as a noninvasive and representative biological sample for the upper genital tract. Menstrual tissue contains parts of the functional layer of the endometrium (decidua) and encompasses the specific perimeter of viral/bacterial activity and implantation of the future embryo.

The infertile control group, consisting of females with endometrial biopsy samples, serves to provide a comparative analysis with the infertile group and to demonstrate the potential of using menstrual tissue as a noninvasive biological sample, analogous in representativeness to invasive endometrial biopsy.

Compilation of patient records for the participants in the study

Detailed patient drecords were meticulously prepared for the infertile Bulgarian females in the sample through the strict application of a questionnaire specifically designed for this purpose, which contributed to the formation of the clinical groups in the current study.

The records contained comprehensive information related to the diagnosis of infertility, including: the period and reasons for sterility, hormonal status, exposure to harmful environmental or occupational conditions, lifestyle, history of inflammatory diseases of the urogenital tract, surgical interventions on the anatomical compartments of the female reproductive system, presence/absence of autoimmune diseases and/or other associated conditions, information on conducted clinical, imaging, genetic tests related to reproductive problems, and administered treatments.

During the course of the research, the records were supplemented with the results of the diagnosis of the target bacterial and viral pathogens *(Chlamydia trachomatis, Ureaplasma urealyticum/parvum, Mycoplasma hominis/genitalium, Gardnerella vaginalis, HSV1/2, EBV, CMV, VZV, HHV-6, HHV-7, HHV-8)* and endometrial microbiome. All probands were, of course, promptly informed of their results through our medical collaborators, allowing for the application of appropriate therapy.

2. Optimization of molecular biological methodology for isolation of total DNA from biological samples: menstrual tissue and endometrial biopsy.

In the pursuit of identifying the most efficacious DNA extraction methodology for specimens derived from menstrual tissue and endometrial biopsy, a rigorous comparative analysis was conducted among the following three DNA isolation procedures:

Phenol-chloroform DNA extraction method

The phenol-chloroform extraction method was employed as the gold standard for the purpose of executing a comparative analysis and subsequent selection of the methodology with the

best extraction parameters. DNA extraction was performed according to an internal laboratory protocol.

Commercial extraction utilizing the AmpliSens Ecoli s.r.o Slovac Republic kit:

Total DNA isolation from menstrual tissues and endometrial biopsies was performed employing a commercially available kit from AmpliSens Ecoli s.r.o Slovac Republic. Adherence to the kit's instructions was unwavering. Subsequent to extraction, DNA yield was ascertained through meticulous spectrophotometric quantification, and the extracted matrices were subjected to NanoDrop technology for precise evaluation of DNA concentration and purity. It is noteworthy, however, that the outcomes obtained from this method were not deemed satisfactory.

Optimization of commercial extraction esing the AmpliSens Ecoli s.r.o Slovac Republic kit

To enhance the efficiency of total DNA isolation from biological specimens using the commercial kit from AmpliSens Ecoli s.r.o Slovac Republic, two optimizing modifications were introduced to the original DNA isolation protocol. These modifications aimed to achieve the highest possible yield of high-quality, purified genomic (human) and exogenous (bacterial/viral) DNA. Firstly, 15mg/µl of Glycogen (Ambion, Life Technologies, USA) was used as a DNA matrix carrier, added simultaneously with the sorbent during the standard procedure to the previously lysed endometrial cells. Additionally, two extra washing steps with washing solutions were introduced after the completion of the standard procedures included in the commercial kit. These supplementary steps involved the addition of 250µl of 75% EtOH, followed by centrifugation for 30 seconds at 10,000 rpm, repeated twice.

The extraction parameters proved to be excellent, matching the data obtained from the control phenol-chloroform extraction. Due to its swifter, safe, and straightforward DNA isolation, the optimized method for commercial DNA extraction using the AmpliSens Ecoli s.r.o Slovac Republic kit was preferred in this study.

3. Introduction of molecular biological methodology for amplification of Chlamydia trachomatis and CD housekeeping gene.

The primary goal in designing primers for the amplification of a target region of the *Chlamydia trachomatis* genome (364 b.w.) was to achieve the highest sensitivity and specificity for pathogen identification. After a literature review, database analysis and applied virtual PCR, a pair of primers was designed that were complementary to specific sequences in the *Chlamydia trachomatis* cryptic plasmid *pLGV440.* The *pLGV440* was repeatedly genome amplified and showed a very high degree of conservation. These characteristics are essential for achieving high analytical sensitivity and for selecting target sequences for primer hybridization in bacterial genomes with typical high mutational variability.

A multiplex variant of the PCR reaction with two pairs of primers was developed to simultaneously amplify a target region of the *Chlamydia trachomatis* genome and a CD housekeeping gene. The purpose of simultaneous amplification in a single reaction is NOT only

to check for the presence of sufficient cells, but also to avoid false-negative results due to PCR inhibition.

Optimizations were conducted to determine the most suitable primer hybridization temperature by using gradient PCR over a wide temperature range (55-64 °C), focusing on GC percentage, the risk of self-pairing, primer dimerization, heteroduplex generation, etc. The optimal hybridization temperature for both primer pairs was determined to be 60 °C, at which both PCR products were perfectly amplified without non-specific amplifications, resulting in the highest fragment resolution (Fig. 1). High diagnostic performance was achieved for the identification of *Chlamydia trachomatis* and the final opimal PCR program was generated.

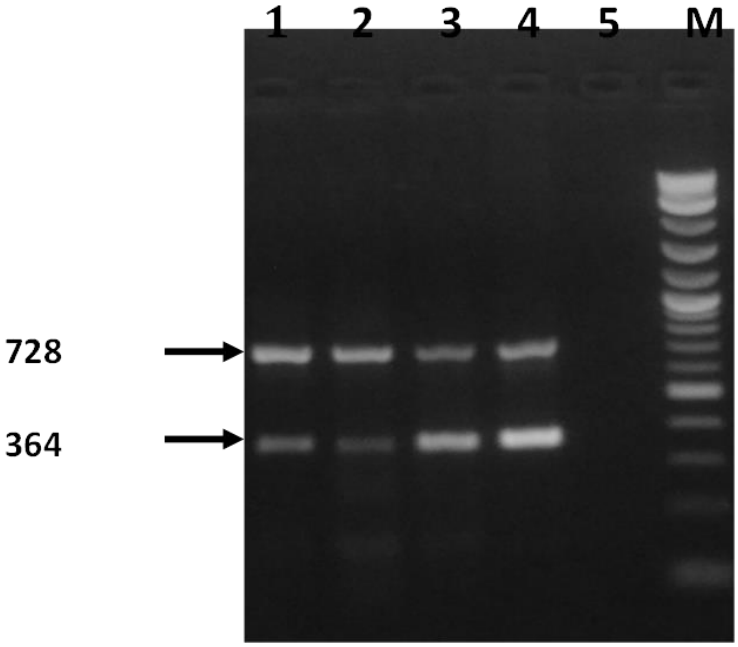


Fig.1. Positive result for *Chlamydia trachomatis* on agarose gel. Two PCR products are visualized:

- **A fragment of the CD gene, used as housekeeping gene -728 base pairs (b.p.).**
- **Chlamydia trachomatis genome fragment - 364 b.p.**
- **Chlamydia trachomatis positive samples /external control/ and presence of CD gene fragment: /1-4 start/.**
- **Negative control /5/.**
- **Sizing marker /M/.**

4. Determining the overall infection rate of the target viral-bacterial panel within the clinical groups.

All samples were analyzed for the presence of the target pathogens *Chlamydia trachomatis, Ureaplasma urealyticum/ parvum, Mycomplasma hominis/genitalium, Gardnerella vaginalis, HSV1/2, EBV, CMV, VZV, HHV-6, HHV-7, HHV-8*. The results of molecular-infectious studies have been reported in 3 publications [1-3].

In 61.1% of the clinical infertile group (180 infertile women tested with menstrual tissue), a positive infection status for the target bacterial-viral panel was detected (48.8% with bacterial infection and 22.2% with viral infection).

The control infertile group (65 infertile patients tested by endometrial biopsy) demonstrated 64.53% positive cases, with 49.23% having bacterial infection and 15.3% having viral infection.

The healthy control group (90 women without reproductive failure or infertility associated diseases tested with menstrual tissue) demonstrated 100% negative status with respect to the target diagnostic panel. This clearly indicates a negative impact of target pathogens on female infertility.

Similar percentages of positive infections in the infertile group tested with menstrual tissue sample (61.1%) and in the control infertile group tested with endometrial biopsy sample (64.53%) and their percentage distribution between viral and bacterial fraction, demonstrate the credability of non-invasive menstrual tissue as a representative sample for the upper female genital tract and confirm the potential of using the same instead of invasive endometrial biopsy.

5. Determination of clinicopathological status and species-specific incidence of infection/co-infection with the study target microorganisms *(Chlamydia trachomatis, Ureaplasma urealyticum/ parvum, Mycomplasma hominis/genitalium, Gardnerella vaginalis, HSV1/2, EBV, CMV, VZV, HHV-6, HHV-7, HHV-8)* in menstrual tissue of infertile females (clinical infertile group).

Clinical and pathological data associated with infertility in probands infected with the target bacterial/viral pathogens in the clinical infertile group.

Among all indivisuals who were positive for the target bacterial/viral infections, 49% had a history of miscarriage, 27.7% had a single miscarriage and 72% =/>2 miscarriages. In 11.1% of the infected infertile clinical group, the miscarriages occurred in the preembryonic stage (up to 5th gestational week), in 61.1% in the embryonic stage (5th-10th gestational weeks) /early miscarriages/ and in 27.7% in (16th-27th gestational weeks) /late miscarriages/.

An unexpected observation was the presence of a history of miscarriages in 64% of the probands with negative infection status. They may have had a positive infectious status at the onset of miscarriage, but none was reported at the time due to administered therapy or lack of current viral reactivity.

Difficulty in conceiving was reported in 35.7% of patients positive for the target bacterial-viral infections.

Autoimmune diseases were recorded in 19.4% of all probands examined and in 20% of those infected. In the group of infected patients with history of miscarriage, 33.3% were diagnosed with autoimmune diseases, while in the group with difficulty in conceiving, 7.14% were diagnosed with autoimmune diseases (Table 2).

Various comorbidities (predominantly associated with infertility) were reported in 13.8% of all examined patients with reproductive issues in the clinical infertile group, 15.5% of the entire

group were active smokers, but 11.1% of these were concentrated specifically among infection-positive probands.

Table 2. Clinical and pathological data associated with infertility in probands infected with target bacterial/viral pathogens - clinical infertile group.

Characteristics	Infected probands with a history of miscarriages
With miscarriage/miscarriages	49,0%
Single miscarriage	27,7%
2 or more miscarriages	72,3 %
Up to 5th gestational week	11,1%
5th-10th gestational week	61,1%
16th-27th gestational week	27,7%
Failed IVFs	20,4 %
Autoimmune diseases	33,3 %
Infertility period	1,5-7,5 г.
	Infected probands with difficulty in natural conception
Infertility period	1,5-15 г.
Failed IVFs	3,57 %

Species-specific prevalence of bacterial and viral infections in the infertile clinical group positive sample.

The prevalence rates of active infection with bacterial and viral pathogens, (calculated as a percentage of all positive bacterial and viral cases) are presented in Table 3.

Table 3. Species-specific prevalence of bacterial and viral infections in the infertile clinical group positive sample.

Pathogen	Number of positive inf. cases in the sample	Percentage %
Ch.tr.	0	0 %
MHO	2 (88)	2,27 %
MGE	0	0 %
UUR	2 (88)	2,27 %
UP	54(88)	61,36 %
GV	61(88)	69,31 %
HSV1	0	0 %
HSV2	1 (40)	2,5 %
EBV	16 (40)	40 %
CMV	3 (40)	7,5 %
VZV	0	0 %
HHV-6	4 (40)	10 %
HHV-7	17 (40)	42,5 %
HHV-8	0	0 %
Bacterial infection	88 (110)	80 %
Viral infection	40 (110)	36,3 %

Co-infection status was observed in 39.9% of all positive for infections cases. The most frequent variants were: *UP/GV - 53.48 %; HHV-7/GV - 11.62 %, EBV/UP - 11.62 %*. The following combinations were detected with an equivalent frequency of 2.32 %: *HHV-7/UP, HHV-7/UP/GV, CMV/GV, HHV-6/GV, HHV-6/GV/UP, EBV/GV, MHO/UP/GV, UUR/ GV, EBV/MHO/UUR/UP/GV, EBV/HHV-7/UP*.

Interpretation and summary of the molecular infectious data in the infertile clinical group.

Chlamydia trachomatis was NOT detected in our target sample of probands, which contradicts the data indicating a prevalent occurrence of genital Chlamydia trachomatis infections [7-8].

In the present study, the reported findings are attributed to the selection of probands with negative cervicovaginal *Chlamydia trachomatis* status, which ensured the absence of menstrual tissue contamination. Such a selection criterion is missing in almost all other studies.

Another reason for the observed difference is that some of the cited literature data pertain to studies conducted on biological material - cervicovaginal fluids, which are representative of lower genital tract infection and not indicative of infection in the endometrium and its association with infertility.

A significant point contributing to the discrepancy identified in results is also the medical history we obtained, indicating antibiotic treatment in a substantial number of patients with infertility. Most of the examined female patients who tested positive for *Ureaplasma parvum, Gardnerella vaginalis*, or variants of coinfection involving *Ureaplasma parvum/Gardnerella vaginalis* (either in combination with viral factors *like EBV, HHV-6/7, CMV*, or without) had a positive history of chlamydial infection treatment and elevated *IgG* chlamydia antibodies.

Despite the fact that in the probands studied, the clinical complications observed could NOT be attributed to a direct ongoing infection with the bacterium Chlamydia trachomatis, the cause of reproductive failure in these cases is complex and, in conclusion, the infectious etiology has a long-term impact. It is highly likely that infections with pathogens such as Chlamydia trachomatis have already been treated and eliminated at the present moment, but their consequences may still play a role as an etiological factor in the intricate scenario of unexplained infertility.

Mycoplasma hominis and Ureaplasma urealyticum were detected in 2.27% of bacterially infected menstrual tissues (Table 3), which is not consistent with the results of Michou and team, who reported that 13.7% of menstrual tissues of infertile women were positive for *Mycoplasma hominis* and 18.3% for *Ureaplasma urealyticum* [7]. The reported in the literature rates of *Ureaplasma urealyticum* in 37.5% positive sterile women and 17.2% in women who underwent in vitro fertilization exceeded by a considerable margin the detected incidence in the present dissertation of 2.27% (Fig. 2) [9-10]. The detected frequencies of *Ureaplasma urealyticum* 54.3% and *Mycoplasma hominis* 30.4% in infertile patients hospitalized for misscariage, and *Ureaplasma urealyticum* (25%) and *Mycoplasma hominis* and *group B Streptococcus* (11.1%) in gestational postabortive tissues of Tavo, Allanson and group do not match the reported data in the present study [11-12].

The reported differences are considered normal due to apparent disparities in the research design, particularly regarding the type and purity of the acquisition of biological materials.

Literature data for comparison primarily originate from the lower genital tract, even in women with reproductive failure, which contradicts our thesis and the study conducted on samples from menstrual tissue and endometrial biopsies. On the other hand, in publications reporting research conducted on the upper genital tract, there is no determination of the lower genital infectious status, which likely inflates the reported frequencies due to potential contamination of menstrual samples from the vagina.

The well-known high vaginal prevalence of genital mycoplasmas and the risk of contamination of endometrial samples led us to include only women with a negative vaginal infectious status regarding *Ureaplasma urealyticum* and *Mycoplasma hominis*, as well as other target pathogens, in the clinical groups of the present study. This specificity serves as a unique sieve and refines our sample concerning data from the lower genital tract, which would contradict the assessment of the direct association between genital mycoplasmas and infertility.

Mycoplasma genitalium

Mycoplasma genitalium Infection was not detected in the study.

The negative status of the examined menstrual tissues and endometrial biopsies regarding *Mycoplasma genitalium* is expected since this pathogen is typically sexually transmitted, and the probands have been extensively screened for such infections in the context of their reproductive issues.

Regarding a positive association of *Mycoplasma genitalium* infection with preterm birth, miscarirage, and infertility, both conclusive and contentious evidence exists from various reports [13].

An important characteristic is the absence of pathological conditions such as pelvic inflammatory disease and clinical cervicitis in the examined patients, where a significantly higher frequency of infection with the bacterium is reported [14-15]. This specificity and the low frequency of Mycoplasma genitalium, ranging from 4% to 5.9% in large case-control studies, indicate that the absence of *Mycoplasma genitalium* in 180 infertile Bulgarian patients is entirely normal.

Ureaplasma parvum

A positive infection status of menstrual tissues for *Ureaplasma parvum* was detected in 30% of the probands tested or 61.36% of all positive bacterial samples (Fig. 2).

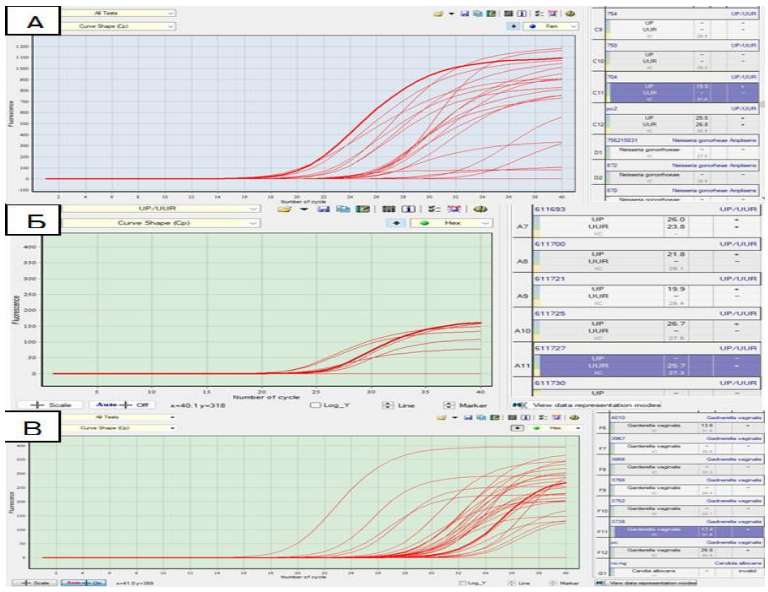


Fig. 2. Positive Real-Time PCR result for clinically significant active infection with:
A. Ureaplasma urealyticum
B. Ureaplasma parvum
C. Gardnerella vaginalis

The identified colonization with *Ureaplasma parvum* in the placenta, amniotic fluid, fetal blood, umbilical cord, fetal and neonatal lung tissues, cerebrospinal fluid, as well as in gastrointestinal aspirates, indicates the pathogen's facile and extensive invasion into the tissues of the female genital tract, akin to what was observed in the current study regarding the endometrium

[16-21]. Postmortem endometritis with septicemia, chorioamnionitis, and a direct impact on adverse pregnancy outcomes are absolutely proven to be associated with *Ureaplasma parvum*, as observed in the present scientific work [22].

The obtained results in the study support the conclusion drawn from other clinical investigations that *Ureaplasma parvum* infection in the placenta and endometrium is associated with stillbirth, misscariage, preterm birth, and low birth weight. All of these scenarios, resulting in infertility, are present in the medical history of reproductive failure of individuals with positive *Ureaplasma parvum* infections in their menstrual tissues. The current data regarding the high prevalence of *Ureaplasma parvum* in infertile females with a history of recurrent miscarriages correlate with the literature, which suggests that the bacterium is more frequently isolated from patients with a history of recurrent miscarriages than from normal pregnant and non-pregnant women [23].

Some probands infected with *Ureaplasma parvum/urealyticum, Gardnerella vaginalis, Mycoplasma hominis* do not exhibit physiological obstructions or complications in the genital tract. In these cases, infertility is likely a result of the direct infection of the zygote by an infected spermatozoon, and the positive infectious endometrial status is a recent event that has not yet triggered inflammatory complications. The current observations support the hypothesis of bacterial invasion via vertical transmission through infected seminal fluid, which aligns with the existing literature [24].

Despite the well-documented causal link between *Ureaplasma parvum* infection and serious reproductive issues, there is no established diagnostic timely approach to identify it in the female endometrium and the upper genital in general. The severity of the late consequences of *Ureaplasma parvum* infection (chorioamnionitis, premature rupture of membranes, preterm birth, fetal inflammatory syndrome, fetal death) underscores the value of non-invasive screening through menstrual tissue even further.

Gardnerella vaginalis

Positive *Gardnerella vaginalis* infection was detected in 33.8% of the infertile clinical group (constituting 69.31% of all positive bacterial menstrual tissues). The high prevalence confirms the link between the proinflammatory state of the endometrium and adverse pregnancy outcome through the clinical data of infected patients (primarily biochemical conceptions, early and late miscarriages and one stillbirth).

The high percentage of *Gardnerella vaginalis* in endometrial tissues (Fig. 2) and its absence in control vaginal specimens present the ascending nature of this microorganism, stemming from past *Gardnerella vaginalis*-associated bacterial vaginosis. Bacterial vaginosis defines a 50% risk of ascending infection to the upper genital tract and establishment of a structured polymicrobial biofilm of *Gardnerella vaginalis* on the endometrium and fallopian tubes, as hypothesized for part of the infertile clinical group [25].

The colonization of the endometrium with *Gardnerella vaginalis* adversely affects pregnancy outcome, as evidenced in a sample of infertile women with extensively infected menstrual tissues. Current data align with reported *Gardnerella vaginalis*-dominant endometrial colonization and absence of parallel infection with other pathogens, suggesting an autonomous negative impact of *Gardnerella vaginalis* on fertility parameters [26]. The mass bacterial invasion in the sample indicates endometrial colonization and subsequent proinflammatory reaction, followed by immune imbalance, as the leading mechanism in cases of compromised pregnancy,

failed implantation, miscarriage and premature delivery in the probands studied, which has also been reported by several other teams [27].

The reported data of presence of 53.48% *Gardnerella vaginalis/Ureaplasma parvum* coinfection in the infected menstrual tissues but pure cervicovaginal status directly confirms the tropism of the pathogens to the upper genital tract. The hypothesis of direct vertical transmission to the endometrium via infected seminal fluid is of course entirely acceptable.

HHV-6

The incidence of *HHV-6* is 10% in the infertile clinical group, which is significantly lower compared to the literature data reporting 40% positive cases in women with idiopathic infertility (Fig. 3).

It is believed that *HHV-6* infection of endometrial epithelial cells, a condition that has been demonstrated to disrupt decidualization [35], is associated with some of the cases of miscarriages in probands infected with the virus in the present sample. *HHV-6* is likely to have downregulated the expression of markers for decidualization *(HLA-G23, MUC1),* which are critical for endothelial cell differentiation, implantation, angiogenesis, trophoblastic cell growth, and immune regulation during early pregnancy, as reported [35-36].

HHV-6 virus does not impact sperm parameters, but it has been reported in 70% of males with normal semen analyses and 66.3% of men with fluctuations, indicating vertical transmission through the partner as the primary mode of infection of the female endometrium [37].

The lower frequency detected in this study is likely due to the unexplored population index of male sperm carriers of the virus, which serves as a major transmission reservoir for the infection.

HHV-7

In the infertile clinical group, a positive *HHV-7* infection was detected in 42.5% of cases, which is the highest frequency of all *HHVs* positive menstrual tissues (Fig. 3). The surprising findings suggest a potential link between active asymptomatic HHV-7 infection as a factor and/or cofactor in the female endometrium and the complex clinical picture of idiopathic female infertility.

Such data are significant *as HHV-7* shares morphological similarities with *HHV-6,* which has documented negative effects on reproduction, and both viruses are concurrently involved in the pathogenesis of other diseases [28].

Nearly 100% of the population becomes infected with *HHV-7* during infancy and is known to remain in a latent state thereafter. A logical hypothesis for the established significant frequency of *HHV-7* in the infected menstrual tissues of infertile probands is assumed to be an undergone reactivation due to hormonal and alternative exogenous and/or endogenous stimuli. Given the lack of negative influence via *HHV-7* on spermatogenesis and semen parameters and its absence in seminal fluid, the hypothesis of vertical transmission via infected spermatozoa does NOT apply.

Data regarding the role of HHV-7 in infertility are scarce, insufficient and sporadic. Although there are reports of subclinical viremia with *HHV-7* in infertile women and negligible prevalence of virus in cervical fluids, extensive future studies are needed to establish/reject an association with a history of infertility.

CMV

Among the many viral factors implicated in pregnancy loss, maternal infections caused by *CMV* and EBV are considered to be significant contributers. Given data of massive placental infections with *EBV* 22.5% and *CMV* 30% in females with recurrent miscarriages (confirmed by immunohistochemistry and "in situ" hybridization [31]), such a scenario cannot be categorically ruled out for the present sample of Bulgarian females. Placental infection with *EBV* and *CMV* occurs through the uterine cavity of the pregnant woman, respectively the endometrium.

The detected *CMV* incidence of 7.5% in infected menstrual tissues corresponds to the reported 11.5% in cervical and placental tissue samples (Fig. 3) [31]. *CMV* successfully proliferates in the endometrium and placenta during pregnancy, replicating in trophoblast, epithelial, stromal cells, macrophages and fetal tissues. In cases of *CMV*-infected endometrium, there is subsequent massive viral invasion into the mentioned compartments, leading to spontaneous pregnancy loss due to an inflammatory response, which could be a reason for adverse outcomes in the analyzed group [38]. Apart from the mechanism of cervical reactivation and ascending infection to the endometrium, an alternative pathway for endometrial *CMV* colonization is through infected seminal fluid [39-40]. The theory is supported by the reported higher incidence of *CMV* in seminal fluid of partners of patients with a history of recurrent miscarriages compared to the control group ($p<0.05\%$) [41].

EBV

The detected incidence of *EBV* in infected menstrual tissues is significant - 40% (Fig. 3) and aligns with published data on *EBV* prevalence (mainly in endocervical samples) ranging from 8.9% in a Swedish sample, 18-19% in Italian patients to 38% again in European females [29-30].

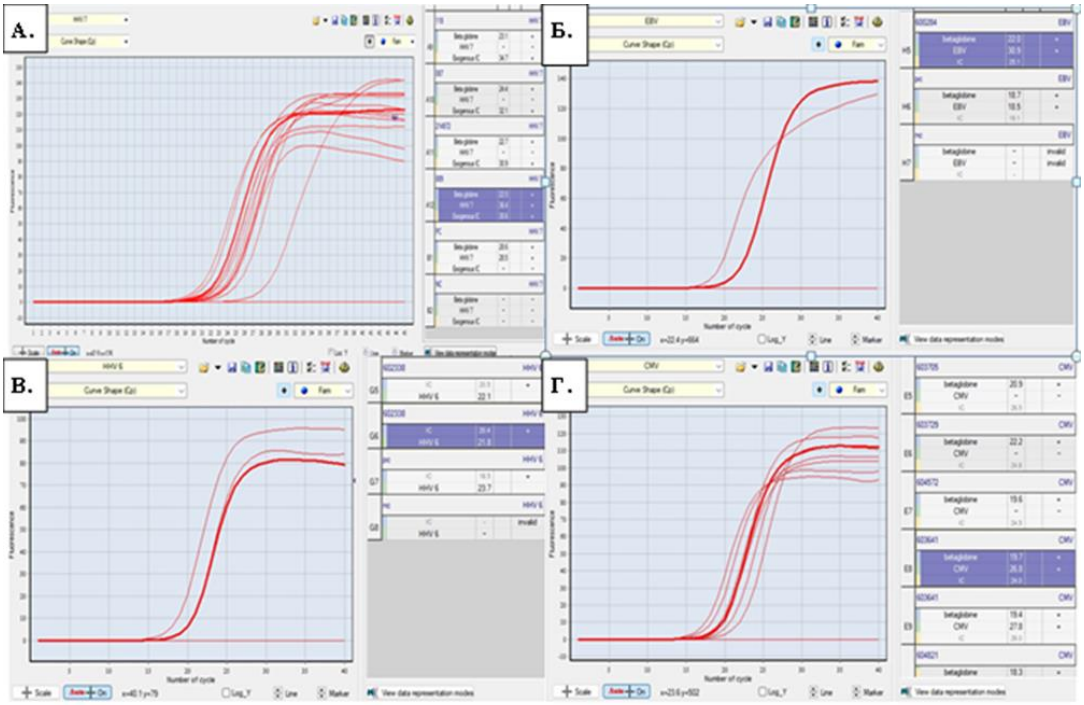


Fig. 3. Positive Real-Time PCR result for clinically significant active infection with:

A. *Human herpesvirus 7 (HHV-7)*
B. *Epstein-Barr virus (EBV)*
C. *Human herpesvirus 6 (HHV-6)*
D. *Cytomegalovirus (CMV*

Due to the high incidence of *EBV* 40% in menstrual tissues, which alignes with scientific data, it is assumed that the endometrium of infertile probands is enriched with the pathogen and plays a significant role in cases of recurrent miscarriages or earliest ones associated with immunological rejection and impaired implantation.

Endometrial *EBV* colonization is a function of reactivated latent infection or intrauterine transmission via infected spermatozoa. The potential vertical transmission via the partner, as also observed in the current sample, is supported by evidence of *EBV* DNA in seminal fluid of infertile males in 0.4% , 16.8%, 40.6% and induced leukocytospermia [32-34].

HSV1/2

In the study group, *HSV2* was detected with a minimal frequency of 2.5% (Table 3), with positive patients having a history of early miscarriages.

The results obtained do not align with published data on the presence of *HSV1/2* DNA in menstrual tissue in 55% of females and 24% of males with infertility and unsuccessful IVF procedures ($p = 0.0086$) [42].

It is believed that *HSV1/2* reactivation in endometrium leads to increased NK cell activity, causing *Th1/Th2* cytokine imbalance, thrombogenic effect, apoptosis in decidual and trophoblastic tissues, resulting in pregnancy loss [43-45]. The direct negative effect of *HSV* on morphology and motility of spermatozoa in males again involves the thesis of direct infection of the newly formed zygote by an affected spermatozoa, which is associated with subsequent pregnancy loss [46].

The high literature incidence of endometrial infections with *HSV1/2* and its association with first trimester pregnancy loss underscore the need for its detection using precise and sensitive molecular techniques.

Coinfection

The results in this study show that the most frequent coinfection variants are as follows: *Ureaplasma parvum/Gardnerella vaginalis* - 53.48%; *HHV-7/Gardnerella vaginalis* - 11.62%, *EBV/Ureaplasma parvum* - 11.62%. It is presumed that all variants of coinfections, even rare ones, exert an influence on the complex etiology of reproductive failure. The most probable pathological mechanism involves triggering additional vulnerability of the endometrium, disruption of local balance and immunity, inflammatory changes in the tissues of the upper genital tract.

6. Determination of species-specific frequency of infection/coinfection with the target for the study microorganisms *(Chlamydia trachomatis, Ureaplasma urealyticum/ parvum, Mycomplasma hominis/genitalium, Gardnerella vaginalis, HSV1/2, EBV, CMV, VZV, HHV-6, HHV-7, HHV-8)* in endometrial biopsies from infertile females (infertile control group).

The control group of infertile females tested for the target infection panel on endometrial biopsy specimens consisted of 65 patients (22-49 years). They were selected based on history of early and/or late recurrent misscariages, absence of autoimmune and other infertility associated

diseases. Negative status with respect to the target bacterial-viral panel in the lower tract was also a selective criterion in this group.

The purpose of the control group thus selected is to assess the interchangeability of invasive endometrial biopsy with a noninvasively acquired sample - menstrual tissue.

All endometrial biopsies from infertile Bulgarian females were tested for the target bacterial-viral panel *(Chlamydia trachomatis, Ureaplasma urealyticum/ parvum, Mycomplasma hominis/genitalium, Gardnerella vaginalis, HSV1/2, EBV, CMV, VZV, HHV-6, HHV-7, HHV-8)*. A healthy control group was not used due to the invasive nature of endometrial biopsy sampling.

The frequencies of detected bacterial and viral infections, expressed as a percentage of all bacterially and virally infected probands, are shown in Table 4. *Chlamydia trachomatis*, *Ureaplasma urealyticum, Mycomplasma hominis/genitalium, HSV1/2, VZV and HHV-8* infections were not detected.

Table 4. Positive infectious factors and co-infection variants in the control infertile group of probands tested through endometrial biopsy.

Infectious Factor	Frequency	Viral-bacterial variants of coinfection
EBV*	**30%**	---
CMV*	**30%**	• *CMV* + moderately expressed aerobic dysbacteriosis and dominant bacterial fraction: *Streptococcus spp.*
		• *CMV* + dysbacteriosis with mixed etiology: *Lachnobacterium spp. + Clostridium spp., Enterobacteriaceae and Streptococcus spp.*
HHV-6*	**20%**	• *HHV-6* + highly expressed anaerobic dysbacteriosis with a dominant bacterial fraction: E*ubacterium spp.*
HHV-7*	**20%**	---
Gardnerella vaginalis*	***31,25%***	• ***Gardnerella vaginalis (dominant) + anaerobic dysbacteriosis: Prevotella bivia, Porphyromonas spp.***
		Gardnerella vaginalis (dominant) + anaerobic dysbacteriosis a: Mobiluncus spp., Corynebacterium spp., Eubacterium, Atopobium vaginae
		Gardnerella vaginalis (dominant) + aerobic dysbacteriosis: Staphylococcus spp., Enterobacteriaceae spp.

Ureaplasma parvum*	***3,12%***	***---***

***Following individual therapy (1.5-3 months), complete bacterial and/or viral clearance of the upper genital tract is observed. Retest was performed again on endometrial biopsies for therapeutic monitoring purposes.**

7. Determination of endometrial microbiome status in endometrial biopsies from infertile females /infertile control group/.

In addition to the target bacterial-viral panel, endometrial biopsies were tested to assess endometrial status (anaerobic, aerobic bacterial and commensal fraction microorganisms). **This allowed for determination of the incidence of endometrial dysbiosis in the infertile control group**.

The condition of anaerobic and aerobic dysbacteriosis was found in 53.33% and 27% in all endometrial samples, respectively. Presence of dysbacteriosis with mixed etiology was identified in 13.33%, while 7% exhibited a dysbiotic state without the target bacteria and in the complete absence of the commensal fraction of microorganisms of *p. Lactobacillus* (Fig. 5.).

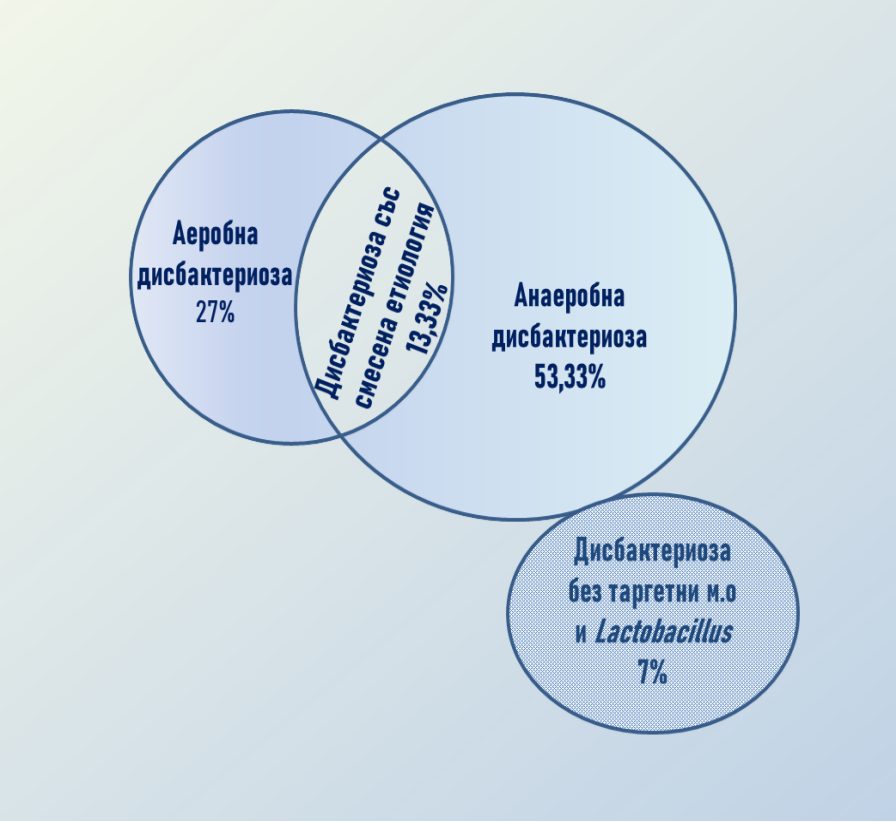
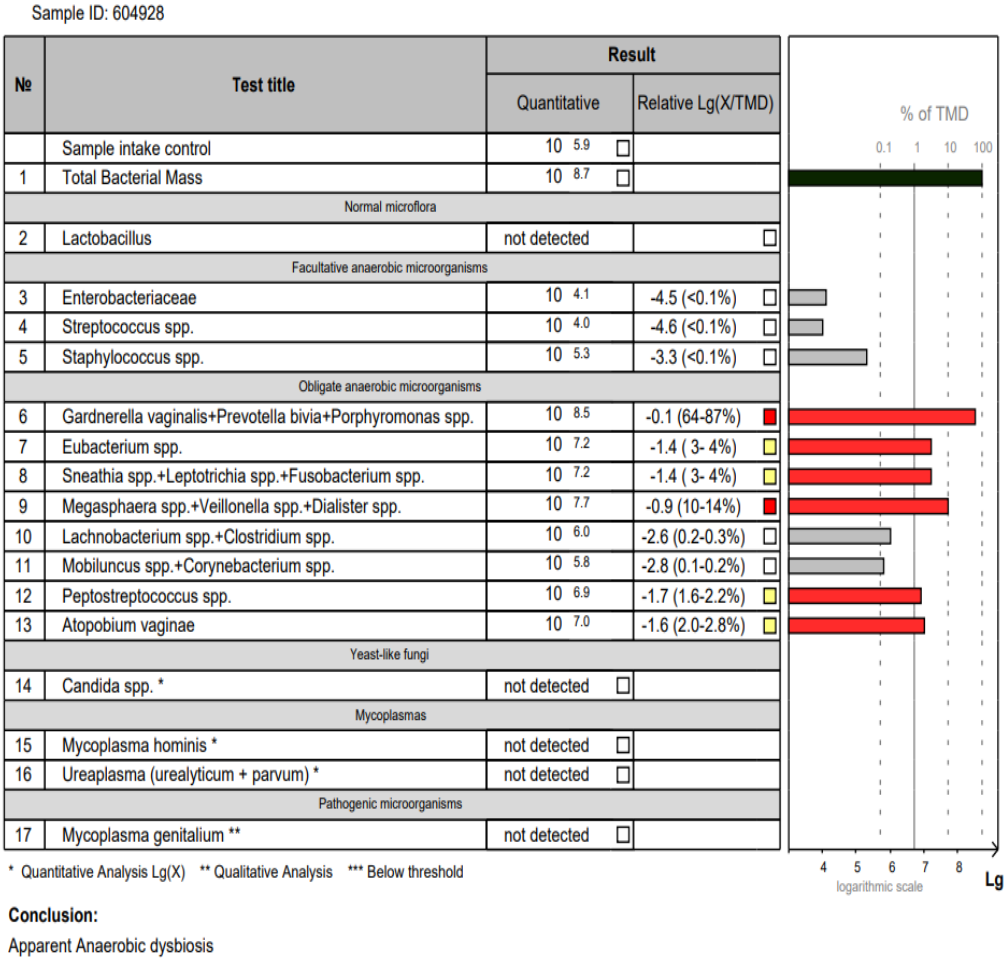


Fig. 5. Types of dysbacteriosis resulting from unbalanced bacterial growth, endometrial colonization, and subsequent inflammation.

- **<u>Anaerobic dysbacteriosis</u>** */positive bacterial findings/: Mobiluncus spp., Corynebacterium spp. Eubacterium spp., Megasphaera spp., Veillonella spp., Dialister spp. Eubacterium spp. , Gardnerella vaginalis, Prevotella bivia, Porphyromonas spp., Peptostreptococcus spp., Atopobium vaginae, Lachnobacterium spp., Clostridium spp.*
- **<u>Aerobic dysbacteriosis</u>** */positive bacterial findings/: Streptococcus spp., Enterobacteriaceae spp., Staphylococcus spp.*

The detected anaerobic and aerobic bacteria in endometrial biopsies correlate with the results of high-tech research on the endometrial microbiome [47-48]. The most common variant of *Gardnerella vaginalis* dominant anaerobic dysbacteriosis is in combination with *Prevotella bivia, Porphyromonas spp*. and the second *with Mobiluncus spp. Corynebacterium spp. Eubacterium* and *Atopobium vaginae* (Fig. 6). *Staphylococcus spp*. and *Enterobacteriaceae spp.* are found predominantly in cases of aerobic dysbacteriosis.



Sample ID: 604928

№	Test title	Result	
		Quantitative	Relative Lg(X/TMD)
	Sample intake control	$10^{5.9}$	
1	Total Bacterial Mass	$10^{8.7}$	
	Normal microflora		
2	Lactobacillus	not detected	
	Facultative anaerobic microorganisms		
3	Enterobacteriaceae	$10^{4.1}$	-4.5 (<0.1%)
4	Streptococcus spp.	$10^{4.0}$	-4.6 (<0.1%)
5	Staphylococcus spp.	$10^{5.3}$	-3.3 (<0.1%)
	Obligate anaerobic microorganisms		
6	Gardnerella vaginalis+Prevotella bivia+Porphyromonas spp.	$10^{8.5}$	-0.1 (64-87%)
7	Eubacterium spp.	$10^{7.2}$	-1.4 (3- 4%)
8	Sneathia spp.+Leptotrichia spp.+Fusobacterium spp.	$10^{7.2}$	-1.4 (3- 4%)
9	Megasphaera spp.+Veillonella spp.+Dialister spp.	$10^{7.7}$	-0.9 (10-14%)
10	Lachnobacterium spp.+Clostridium spp.	$10^{6.0}$	-2.6 (0.2-0.3%)
11	Mobiluncus spp.+Corynebacterium spp.	$10^{5.8}$	-2.8 (0.1-0.2%)
12	Peptostreptococcus spp.	$10^{6.9}$	-1.7 (1.6-2.2%)
13	Atopobium vaginae	$10^{7.0}$	-1.6 (2.0-2.8%)
	Yeast-like fungi		
14	Candida spp. *	not detected	
	Mycoplasmas		
15	Mycoplasma hominis *	not detected	
16	Ureaplasma (urealyticum + parvum) *	not detected	
	Pathogenic microorganisms		
17	Mycoplasma genitalium **	not detected	

* Quantitative Analysis Lg(X) ** Qualitative Analysis *** Below threshold

Conclusion:

Apparent Anaerobic dysbiosis

Fig. 6. Anaerobic dysbacteriosis with dominant bacterium *Gardnerella vaginalis* in combination *with Prevotella bivia, Porphyromonas spp*. Complete absence of commensal fraction: *Lactobacillus.*

Dysbiotic conditions in the study are characterized by a decreasing proportion of *p. Lactobacillus* to varying degrees, as also reported in the literature [49]. Teams focusing on

bacterial interactions have demonstrated that *p. Lactobacillus* aggregating commensal myctoorganisms is negatively associated with *Gardnerella, Bifidobacterium* and *Atopobium* [48], which holds true for *Gardnerella vaginalis* and *Atopobium vaginae* in the current study.

The drastic reduction or total absence of *Lactobacillus spp*. reported in the study sets the stage for: uncontrolled growth of residual bacterial fractions from past infection, excessive growth of opportunistic bacteria, and increased vulnerability of the endometrium to new infection, proinflammatory response, and immune imbalance. This cascade of events suggests the role of the endometrial microbiome in the successful implantation and pregnancy progression, emphasizing the need for endometrial microbiome testing of all women with reproductive difficulties.

It is worth noting that the lower genital tract samples (cervical-vaginal fluids) in the studied infertile control group showed normocenosis and conventional normocenosis. However, in the endometrium, moderate and severe dysbacteriosis was detected, along with variants of polymicrobial coinfection. This highlights the importance of performing the test on an endometrial sample (menstrual tissue or biopsy).

8. Comparative analysis of clinical and control infertile group data regarding the application of the target viral-bacterial panel on menstrual tissue biological material and endometrial biopsy.

In the control infertile group, 35.47% of the tested patients exhibited negative infectious status, which corresponded to the results of menstrual tissue (38.9%). 64.53% of all endometrial biopsies tested were detected with a positive infectious status (49.23% bacterial and 15.3% viral pathogens). The obtained data once again correlate with the established 61.1% infection-rate in menstrual samples in the infertile clinical group (48.8% bacterial and 22.2% viral). Strikingly, the percentage of concordance in the infection rate found in the clinical and control groups maintained its distribution regarding both viral and bacterial components.

The high comparability of the data regarding the infectious bacterial/viral status in the clinical and control infertile groups`unequivocally demonstrates that the non-invasive menstrual tissue is representative of the upper female genital tract and confirms the feasibility of using it instead of invasive endometrial biopsy.

The negative infectious status reported in 100% of the healthy controls in this study, clearly indicates the negative impact of the target infection panel on female infertility (Table 5).

Table 5. Overall infection incidence for the target viral-bacterial panel.

Clinical group	Negative infectious status	Positive infectious status
Infertile (180)	**38,9%***	**61,1%* (48.8% bacterial and 22.2% viral)**
Control (90)	**100 %**	**0%**
Infertile with endometrial biopsy (65)	**35,47%***	**64,53%* (49.23 bacterial and 15.3% viral)**

***High degree of correlation regarding negative/positive infectious status between infertile clinical and infertile control groups.**

The lower frequency of *Gardnerella vaginalis* in endometrial biopsies, 31.25%, compared to menstrual tissue, 69.31%, is considered a normal finding given the size and heterogeneity of the sample. The endometrial biopsies group (65) is considered relatively small compared to the number of menstrual tissues studied (180). Additionally, in the endometrial biopsies group there is a positive history only for recurrent miscarriages, whereas menstrual tissues include a wide variety of reproductive and infertility-associated health problems. We assume that as the size and heterogeneity of the infertile control group increase, the statistics will change.

Another significant reason for the observed differences is that in the control infertile group, ~50% of the sexual partners of the selected patients underwent testing and are negative for the target bacterial pathogens by microbiological culture (data not presented).

As previously mentioned, ~1/2 of cases of dominant active *Gardnerella vaginalis* infection in the studied endometrial biopsies are presented in a coinfection scenario, primerily involving other predominantly anaerobic bacteria and less frequently a mixed presence of anaerobic and aerobic bacteria. This particular characteristic naturally applies solely to the infertile control group which was tested with endometrial biopsies, since only these were subjected to additional diagnostics of anaerobic, aerobic and commensal bacterial microorganisms in order to verify the endometrial balance.

Ureaplasma parvum was detected in 3.12% of all bacterially infected endometrial biopsies, which is an extremely low percentage compared to the data obtained from menstrual tissue samples, where the detection rate was 61.36%.

Possible explanations for the low incidence of *Ureaplasma parvum* in the control group include once again: negative status for bacterial pathogens in ~50% of probands' sexual partners, sample size and history, as well as strict inclusion criteria for the study.

Chlamydia trachomatis, Mycoplasma hominis/genitalium, and Ureaplasma urealyticum were not detected in endometrial biopsies, which does not confirm published data [50]. On the other hand, *Mycoplasma hominis* and *Ureaplasma urealyticum* were detected at a very low frequency (2.27%) in menstrual tissues. In practice, in both clinical groups these pathogens were either absent or detected in a minimal percentage. Presumably, the reported results are a function of selective inclusion of probands with negative cervicovaginal status and history of therapy for pelvic inflammatory conditions.

Active infection with ***EBV, CMV, HHV-6***, and ***HHV-7*** was detected in 30%, 30%, 20%, and 20% of virus-positive endometrial biopsies and in 40%, 7.5%, 10%, and 42 , 5% of menstrual tissues (Table 6). These differences are likely due to the smaller number of endometrial biopsies examined (65) compared to menstrual tissues (180). If the sample size of endometrial biopsies increases, we may obtain more similar results. However, it is also possible that the differences and the tendency for higher frequencies of *CMV, HHV-6* in the endometrial biopsy group and higher frequencies for bacterial pathogens and *EBV, HHV-7* in infected menstrual tissues may persist. History of probands appears to be an important divergent criterion.

Table 6: Comparative analysis of the application of the target infection panel in clinical and control infertile group

Positive pathogens	Menstrual tissue	Endometrial biopsy	Comments
CMV	7,5%	30%	Sample heterogeneity Anamnesis
EBV	40%	30%	Sample heterogeneity Anamnesis
HHV-6	10%	20%	Sample heterogeneity Anamnesis
HHV-7	42,5%	20%	Sample heterogeneity Anamnesis
Ureaplasma parvum	61,36%	3,12%	Difference in sample Negative vaginal status Negative partner

Gardnerella vaginalis	**69,31%**	**31,25%**	**Difference in sample Negative vaginal status Negative partner**

The reported results hypothesize different associations of certain infections with specific reproductive disorders. For instance, the much higher percentage of *CMV* in endometrial biopsies (40%) versus (7.5%) in positive menstrual tissues suggests that *CMV* is likely primarily associated with miscarriages and less frequently with other reproductive failures. Conversely, the higher percentage of *HHV-7* in menstrual tissues, 42.5%, compared with the 20% detected in endometrial biopsies, indicates a more probable link to conception-related problems than miscarriage. Further studies are needed to support the identified trends, associations and multifactorial constellations.

Furthermore, the sexual partners of the infertile control group of patients were not screened for the viral target pathogens. This creates an additional prerequisite for viral transmission to the upper genital tract and a higher prevalence of *CMV, HHV-6* compared to the established bacterial profile in this clinical group.

The viral pathogens ***HSV1/2, VZV*** and ***HHV-8*** were not detected in the endometrial biopsies examined, whereas only *HSV2* was detected in 2.5% of menstrual tissue of infected infertile probands. The findings aligne with existing literature data indicating a low incidence of *HSV1/2* infection (1.4%-1.7%) and a total absence of *VZV* in endocervical fluids of asymptomatic patients [51]. At the same time, *HSV1/2* was reported at a much higher rate of 55% in menstrual tissues of infertile females in a pilot study by El Borai, which contradicts the present results [42]. It should be taken into account that in the cited study on menstrual tissue, most pathogen prevalence rates were elevated. Due to the limited data in the literature on this type of biological material, the results cited in this thesis, based on the set selective criteria, provide clarity regarding the association/lack of association of target pathogens with specific reproductive issues.

The entirely negative status regarding *VZV* and *HHV-8* is not surprising, as their reported incidence in the female genital tract is evidently very low or absent, despite the potential for upper genital tract colonization and their association with miscarriage and perinatal infection. It is logical that they were absent in both clinical groups because their distribution is geographically and culturally stratified. It is normal for modern Bulgarian European females with access to vaccines and prevention not to be detected. In the present study, we even rule out the possibility of vertical transmission of *VZV* and *HHV-8* via infected seminal fluid, although *VZV* in seminal fluid of infertile males is reported at an incidence of ~3.2% and has a negative effect on spermatogenesis, whereas HHV-8 has no role in abnormal semen parameters.

An essential aspect of the study was the parallel investigation of 15% of female patients using menstrual tissue and endometrial biopsy. The observed negative or positive bacterial-viral findings were absolutely identical in both types of samples.

The reported 100% concordance of molecular infectious results in the shared sample of patients tested with both sample types is impressive. Once again, it unequivocally confirms that noninvasive menstrual tissue, containing portions of the functional endometrium, is representative for assessing the infectious status in the upper genital tract.

Individualized therapy (1.5-3 months) was administered to all patients with bacterial-viral infection tested by endometrial biopsy. We observed complete bacterial and viral clearance of the upper genital tract by retesting with new endometrial biopsies.

Surprisingly, the most satisfying findings for the present study were not the promising molecular infectious data supporting or not certain reproductive-virulence mechanisms or prevalence frequencies of target infectious factors. The most inspiring result is that natural conception was achieved in 97% of patients subjected to adequate and correct therapy. In the remaining 3% of cases, findings of hyperplastic type endometrial polyp were detected by our medical collaborators. Treatment of these patients continues in the right direction.

Based on all the data obtained, an algorithm for diagnosis and dynamic follow-up of bacterial-viral infections in the endometrium and monitoring of the effect of individualized therapy was developed. The promising data and the undeniable medical-practical benefit of the study represent the essential utility factor for females suffering from unexplained infertility.

9. Establishment of a biobank with isolated total DNA matrices from menstrual tissue and endometrial biopsies of Bulgarian females with reproductive failure.

The extraction of total DNA from the target samples for the scientific research activities in the dissertation was performed in double and triple volumes to completely deplete the biological samples: menstrual tissue and endometrial biopsy. A certain volume of the isolated high-molecular-weight total DNA matrices were allocated for the analyses in the research, including possible replicates. They were stored at -20 ºC after completion of molecular infectious analyses.

The residual DNA matrices isolated from menstrual tissue and/or endometrial biopsies of infertile patients were used to create a biobank. The DNA samples were labeled with a code known only to the lead researcher (a current PhD student) and her direct scientific supervisors. The aim was to properly store (at -70 °C), the well-characterized samples of Bulgarian females with reproductive failure and accompanying multifactorial medical information associated with the diagnosis of infertility. In this way, the biobank of selected DNA molecules will be available to the PhD student, her scientific supervisors and the formed research groups during her doctoral studies for future experimental and upgrading scientific activities.

Certainly, the DNA matrices frozen at -70 °C were subjected to an annual quality control, which is a one-off. The quality control involved measuring the purity and concentration of the DNA samples using spectrophotometry instruments, including a nanodrop device. Afterwards, PCR was performed to amplify the endogenous internal control *CD*.

SUMMARY OF RESULTS AND CONCLUSION

Various mechanisms explain how changes in intrauterine bacterial and viral fractions can induce infertility and adverse gynecological conditions.

In addition to upper genital tract infection and subsequent inflammatory complications leading to impaired ability to conceive, other virulence mechanisms affect the processes of immunological tolerance at the implantation site, endometrial decidualization, and trophoblast invasion, all directly related to successful implantation and miscarriage. The risk of neonatal infections is also a significant feature of an infected endometrium.

The most likely mechanisms for enrichment of the female upper genital tract (endometrium) with target bacteria and/or viruses in the form of active asymptomatic infection are infection ascent from the lower genital tract, endometrial reactivation of a latent infection, or vertical transmission through a sexual partner who may be clinically significantly infected or in a latent carriage role.

The subject of molecular infectious research in the present PhD thesis were 335 females of Bulgarian origin, tested for the following bacterial-viral panel *(Chlamydia trachomatis, Ureaplasma urealyticum/ parvum, Mycomplasma hominis/genitalium, Gardnerella vaginalis, HSV1/2, EBV, CMV, VZV, HHV-6, HHV-7, HHV-8).* The studied women were divided into three clinical groups based on the type of biological material and history of reproductive failure: infertile; control infertile and control healthy group.

In 61.1% of the infertile group (180 infertile women) tested with menstrual tissue, a positive infectious status for the target bacterial-viral panel was detected (48.8% of all samples tested with bacterial and 22.2% with viral infection).

The infertile control group, consisting of 65 patients with endometrial biopsy, demonstrated 64.53% positive cases (49.23% with bacterial and 15.3% with viral infection). The control infertile group underwent additional analysis for the state of the endometrial microbiome.

The healthy control group, consisting of menstrual-tissue samples from 90 women without reproductive failure or infertility-associated diseases, turned out to be 100% negative for the target diagnostic panel.

The observed similar rates of positive infections in the infertile group (61.1%) tested with menstrual tissue sample and in the control infertile group (64.53%) tested with endometrial biopsy sample demonstrate the representativeness of non-invasive menstrual tissue as a sample for the upper female genital tract and confirm its potential to be used instead of invasive endometrial biopsy.

The reported negative infectious status in 100% of the examined healthy controls, clearly indicates a negative impact of the targeted infectious panel on female infertility.

A characteristic of all clinical groups is the negative cervicovaginal status for the target bacterial-viral panel. This criterion was intentionally included in the study to eliminate the possibility of cervicovaginal contamination of the used endometrial samples (menstrual tissue and endometrial biopsy). The detection of pathogens exclusively localized in the endometrium in this study demonstrates the possibility of upper genital tract infection in the absence of lower tract infection and underscores the need for mandatory targeted infectious diagnosis of the upper genital tract. For this purpose, the use of a non-invasive sample representative of the upper genital tract is crucial in defining the diagnostic screening algorithm.

Gardnerella vaginalis and Ureaplasma parvum bacteria were detected in 69.31% and 61.36% of all positive menstrual tissues, respectively, and 31.25% and 3.12% of all bacterially infected endometrial biopsies. Active infection with *Mycomplasma hominis* and *Ureaplasma urealyticum* was detected at an equivalent frequency of 2.27% in menstrual tissues positive for bacterial pathogens. Active infection with *EBV, CMV, HHV-6* and *HHV-7* was found in 30%, 30%, 20% and 20% of endometrial biopsies positive for viral factors and in 40%, 7.5%, 10% and 42 ,5% of menstrual tissues.

The following bacterial and viral pathogens were not detected in any of the menstrual tissues of the probands studied: *Chlamydia trachomatis, Mycomplasma genitalium, HSV1, VZV*, and *HHV-8. Chlamydia trachomatis, Ureaplasma urealyticum, Mycomplasma hominis/genitalium, HSV1/2, VZV* and *HHV-8* were not detected in endometrial biopsies. Endometrial biopsies were further assessed for the endometrial microbiome status. Anaerobic and aerobic dysbacteriosis were identified in 53.33% and 27% of all endometrial samples, respectively. Such study is lacking on the probands with menstrual tissue, due to the inability to apply the method used to this type of sample.

The infectious fraction identified in the present study did not entirely align with that reported in the existing literature. This finding can be explained by the selection of patients with negative cervicovaginal status. The negative status of our probands for lower genital tract infections excludes contamination brought in from this section. The absence of such a criterion in the reported in the literature incidence of bacterial infection suggests a possible cervicovaginal contamination of endometrial samples and explains the reported higher reported infection rate. Additionally, our probands also had a reported history of frequent antibiotic therapy in the past, which logically explains the lower or absent prevalence of certain pathogens, such as *Chlamydia trachomatis*, etc. Population characteristics also play a role. Heterogeneity in medical history is another key contributor to the findings.

Positive probands from all groups were analyzed in terms of reproductive history to establish a potential link between infectious agent and type of reproductive failure. It was found that *Ureaplasma parvum, Gardnerella vaginalis, CMV* and *HHV-6* were more likely to play a significant role in the occurrence of miscarriages, while other infections were more frequently involved in conception-related issues.

It has been demonstrated that menstrual tissue containing parts of the functional endometrial layer serves as a reliable and informative non-invasive specimen for infectious screening of the upper genital tract. This assertion was substantiated through a comparative analysis between the clinical infertile group using menstrual tissue and the control infertile group with endometrial biopsies. In both groups, we observed an absolute concurrence in the percentage of patients with positive infectious status and their distribution with respect to bacterial or viral etiology. At the level of the species-specific sample, menstrual tissue and endometrial biopsies exhibited some variations in the percentages of pathogens detected. This divergence can be logically attributed to the heterogeneity of the sample population and the diverse medical histories of the subjects.

An additional testament to the representativeness of the sample lies in the fact that, within the jointly tested 15% with endometrial biopsy and menstrual tissue, we have 100% comparability of results. Natural conception was achieved in 97% of patients who underwent adequate and correct therapy for the treatment of infections. The promising data and the undeniable medico-

practical utility of the research are the most serious signifivant acomplishmens of the present dissertation.

A systematic algorithm for diagnosing asymptomatic bacterial and viral infections in the upper genital tract through examination of menstrual tissue/endometrial biopsy and subsequent therapeutic monitoring has been successfully developed.

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CONCLUSIONS:

1. The implemented know-how optimizations in pre-analytical pre-processing, DNA extraction and PCR amplification techniques for *Chlamydia trachomatis* contributed to a higher technological and diagnostic level of efficiency.

2. The similar incidence of positive infections and their distribution conserning bacterial and/or viral components in both the infertile group with menstrual tissue and the control group with endometrial biopsies substantiates menstrual tissue as a valid representative specimen for the upper genital tract analysis (100% concordance rate).

3. The comparative analysis of infection frequency and patients' histories regarding the target bacterial-viral pathogens in this study has established the association of *Ureaplasma parvum, Gardnerela vaginalis, HHV-6, CMV, EBV, HSV2* with infertility, hinting at potential links between pathogen type and clinicopathological presentation.

4. Infectious factors such as *Ureaplasma parvum, Gardnerella vaginalis, CMV* and *HHV-6* exhibited a more significant association with the occurrence of miscarriages when contrasted with other types of infections.

5. The high prevalence of *HHV-7* in menstrual tissue/endometrial biopsy (42.5%/20%) and the limited scientific data predetermine the potential association of asymptomatic endometrial *HHV-7* infection with reproductive failure.

6. The data on the endometrial microbiome in endometrial biopsies revealed a stark difference in the lower genital tract: cervical-vaginal normocenosis contrasting with massive endometrial dysbiosis.

7. The established distinct infectious status in the upper and lower genital tract highlights the need for parallel diagnosis on samples of cervical-vaginal and endometrial origin.

8. The molecular-infectious data obtained from a 15% shared patient sample, encompassing the examination of menstrual tissue and endometrial biopsies, solidified menstrual tissue as a representative sample for the upper genital tract (100% comparability).

9. The high therapeutic efficacy and achieved 97% natural conception rate following upper genital tract clearance of bacterial-viral infections in endometrial biopsies, unequivocally demonstrated the need for infectious screening in the endometrium.

10. The developed systematic algorithm for the diagnosis of „hidden“ asymptomatic infections in the female upper genital tract through the examination of a non-invasive biological sample - menstrual tissue and subsequent therapeutic monitoring yielded very favorable results.

PUBLICATIONS AND SCIENTIFIC EVENTS RELATED TO THE DISSERTATION:

- **Publications related to the dissertation**

1. K. Mesechkova, Bilyana Georgieva, Ivan Sigridov, Ani Miteva, Vanyo Mitev, Albena Todorova "Bacterial and Viral Pathogens Implicated in Female Reproductive Failure Investigated on Menstrual Blood", *C. R. Acad. Bulg. Sci.* , vol. 76, no. 3, pp. 394–406, Mar. 2023. (Impact factor 0,378) Q3
2. Kremena Mesechkova, Anita Kavrakova, Bilyana Georgieva, Ivan Sigridov, Vanyo Mitev, Albena Todorova "Non-Invasive Diagnostics of Reproductive Failure with Infectious Etiology on Menstrual Tissue" J Acta Medica Bulgarica, 50 (3), 2023.
3. Kremena Mesechkova , Anita Kavrakova, Elena Todorova, Bilyana Georgieva, Ivan Sigridov, Vanyo Mitev, Albena Todorova "Role of bacterial and viral infections and co-infections in miscarriages " J Acta Medica Bulgarica, 50 (4), 2023.

- **Scientific Events Related to the Dissertation**

Project with No. D-209/12.12.2018 from the competition „Stimulating Scientific Research in Fields with High Achievements - 2018." Topic: „Investigation of the Most Common Bacterial and Viral Pathogens Implicated in Reproductive Failures in Women using Menstrual Blood as a Target Biological Sample" - (Participant: Kremena Stoycheva Mesetchkova, PhD candidate, Department of Medical Chemistry and Biochemistry - Medical University of Sofia)."

CONTRIBUTIONS

Scientific and Applied Contributions:

1. Experimentally confirmed the biological sample of menstrual tissue as representative of the upper genital tract (endometrium).

2. Proposed a systematic algorithm for the diagnosis of „hidden“ asymptomatic infections in the female upper genital tract through the examination of a non-invasive biological sample: menstrual tissue, followed by therapeutic monitoring.

3. Achieved a high therapeutic effectiveness and successful natural conceptions through the application of the systematic algorithm.

4. Molecular-infectiological data demonstrated the need for non-invasive screening of the endometrium for active bacterial-viral infections and confections associated with infertility, as well as the fact that diagnosing the lower genital tract is not indicative of the overall genital status.

Methodological Contributions:

1. Introduced know-how optimizations for:

- Preprocessing of menstrual tissue biological samples.
- DNA extraction.
- Know-how multiplex PCR method for the identification of *Chlamydia trachomatis*.