Доклади на Българската академия на науките
Comptes rendus de l'Académie bulgare des Sciences
Tome 76, No 3, 2023

BIOLOGY
Microbiology

BACTERIAL AND VIRAL PATHOGENS IMPLICATED IN FEMALE REPRODUCTIVE FAILURE INVESTIGATED ON MENSTRUAL BLOOD

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Received on February 2, 2021
Accepted on March 30, 2021

Abstract

We investigate the impact of bacterial (*Chlamydia trachomatis, Ureaplasma urealyticum/parvum, Mycomplasma hominis/genitalium, Gardnerella vaginalis*) and viral (*HSV1/2, EBV, CMV, VZV, HHV6/HHV7, HHV8*) pathogens, as a potential cause of reproductive failure in women by analysis of menstrual blood. We analyzed DNA extracted from 48 probands selected on the basis of history of infertility. DNA extraction, Real-time qPCR, gel electrophoresis were applied.

In 64.6% of all tested menstrual blood samples of infertile women bacterial and/or viral pathogens were detected. In 41.4% of all tested samples we found bacterial, while in 37.5% viral pathogens. *Ureaplasma parvum* and *Gardnerella vaginalis* were detected in 58.3% and 54.2%, respectively, of the positive for bacterial pathogens samples. *EBV, HHV 7* and *HHV 6* were detected in 38.9%, 55.6%, and, respectively, 11.1% of the positive for viral pathogens samples. Bacterial and viral co-infection was found in 22.6% of all patients.

Chlamydia trachomatis, Mycomplasma hominis/genitalium, Ureaplasma urealyticum, HSV1, HSV2, CMV, VZV and *HHV 8* were not detected in the menstrual blood samples.

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The present study was supported by Grant No. D-209/12.12.2018 of Medical University of Sofia, Bulgaria.

DOI:10.7546/CRABS.2023.03.08

Our study offers new approach for diagnostics of infections in the upper female genital tract by analysis of menstrual blood. The opportunity to detect asymptomatic bacterial and viral infections in female endometrium contributes to reveal the cause for sterility. Our work contributes to clarify the infectious etiology of reproductive failure which is of a great importance for individualized therapy.

Key words: *HHVs*, infertility, menstrual blood

Introduction. The widespread prevalence of sexually transmitted infections implicated in infertility status in women remains a global social and health problem. The detection of bacteria and viruses, resulting in endometrial inflammation is of particular importance. If the endometrial inflammation remains untreated, it could lead to serious complications, as fallopian tube obstruction, ectopic pregnancy, chronic pelvic pain, uncleared prolonged infertility, and even ovarian cancer. The most common method for microbial detection in cervical-vaginal biological specimens is microbial culture. This method detects 70–80% of the infectious pathogens in the lower genital tract in women and has a significantly lower diagnostic effectiveness in the upper genital tract [1].

In order to avoid misdiagnosed endometrial infections it is appropriate to use menstrual blood, as a target biological sample. The menstrual tissue contains shed parts of the functional layer of the endometrium (decidua), as well as blood cells, circulating in the peripheral bloodstream and comprises the particular area of microbial and viral activity and embryonal implantation.

Materials and methods. ***Patients and samples.*** We tested 48 female probands selected on the base of their infertility history: recurrent miscarriages, difficulties in the natural conception, physiological obstruction of particular anatomic sections of female genital tract, in consequence of inflammation or surgical interventions, unsuccessful assisted reproduction procedures, presence of autoimmune disease. All selected 48 infertile women were negative for the target bacterial/viral panel in cervico-vaginal swabs, so we included these probands in the current study to check their bacterial/viral status in the upper genital tract by testing menstrual blood samples. Written informed consent was obtained from all patients prior to genetic testing. The age of the probands is 22–44 years with mean period of infertility 1.2–5 years. About 17% of them are active smokers.

As healthy controls, 25 women without reproductive failure were tested for the target infectious panel in the current research.

Menstrual blood specimens were collected in the heaviest float of the menstrual cycle. As a transport medium for DNA preservation 2 ml 0.5 M EDTA with pH 8.0 was used. Protein digestion with proteinase K (40 µl) for 24 h at 65 °C was performed and sediment was collected after centrifugation for 15 min at 8000 rpm at 4 °C. Total DNA was extracted from the 120 µl sediment using the AmpliSens DNA isolation kit (Ecoli s.r.o, Slovak Republic). Two modifications in the original DNA isolation protocol were made. As a DNA carrier 15 mg/µl

glycogen was added after the preparation with a lysis buffer, simultaneously with adding of the DNA-sorbent component. Furthermore, additional washing steps with 75% ethanol were included resulting in a higher yield and purity of the obtained genomic viral/bacterial DNA. The quality and purity of the obtained total DNA was measured with NanoDrop machine. To prove that the chosen procedure for pre-preparation of the menstrual blood is appropriate, we performed a histological examination of the tested samples, which showed predominance of endometrial cells and a minimal residue of cervical mucus.

The amplification of the target viral and bacterial DNA fragments was performed, using the AmpliSens commercial amplification kits (Ecoli s.r.o, Slovak Republic) based on Real-Time qPCR. Bacterial targets were *Chlamydia trachomatis, Ureaplasma urealyticum/parvum (UP), Mycomplasma hominis/genitalium, Gardnerella vaginalis (GV)* and viral targets were *HSV1/2, EBV, CMV, VZV, HHV6/ HHV7, HHV 8.*

Results. We found positive infectious status with the target bacterial/viral pathogens in 64.58% of our probands' menstrual blood samples.

The *HHVs* testing detected positive *HHV* types in 37.5% of the patients with reproductive problems (58.06% of all infected probands), which is in accordance with the published data for other European populations.

In 14.5% of the studied patients the infection was due to *EBV* (38.8% of the positive *HHVs*) and 20.83% to *HHV-7* (55.56% of the positive *HHVs*). *HHV-6* was detected in only 4.17% of all analyzed cases, or 11.1% of all positive *HHVs* samples.

A positive association between increased levels of NK in blood serum of women with reproductive problems and the presence of a subclinical asymptomatic viremia with *HHVs* in blood was proven. We found positive status of a subclinical asymptomatic viremia with *EBV* and *HHV-6* in a small proportion of our patients with data for NK-increased levels.

Different bacteria types were found in 41.38% of the menstrual blood samples (77.42% of all infected probands). *Ureaplasma parvum* was detected in 29.1% of all menstrual blood samples, or 58.33% of all positive bacterial samples. *Gardnerella vaginalis* was found in 27.08% of all menstrual blood samples, or 54.17% of all positive bacterial cases. A condition of bacterial co-infection was found in 12.5% of all positive cases with bacterial etiology (predominantly *GV* and *UP*), while viral co-infection was found in 5.56% of all positive cases with viral factor (*HHV-7* and *EBV*).

The condition of compound bacterial and viral co-infection was detected in 22.58% from all positive cases with infectious pathology. The most common co-infection conditions involved *HHV-6, HHV-7, EBV* and *GV, UP* bacterial factors. Co- infection condition of *GV/HHV 7* and *UP/EBV* was found in 6.45%. The rest co- infection variants *GV/HHV6, UP/EBV/HHV7, GV/UP/HHV 7* and *UP/HHV 7* were found in 3.22% of all infectious positive samples.

The following pathogens were not detected in any of the probands' menstrual blood samples: *Chlamydia trachomatis, Mycomplasma hominis, Mycomplasma genitalium, Ureaplasma urealyticum, HSV1, HSV2, CMV, VZV*, and *HHV-8*.

All studied healthy controls (25 women without reproductive failure) were negative for the target infectious panel for the current research.

Discussion. Genital infections with Chlamydia trachomatis are the most common bacterial infections in most European countries. Our results show that C. trachomatis was not detected in our patient's cohort, which contradicts to the published data [2].

In our patients with: tubal obstruction and ectopic pregnancy (MB 16, 31, 36, 43, Table 1) active infections of different pathogens were detected: past and recent *Ureaplasma parvum* infection, *EBV/UP* co-infection and *HHV-7*. In the described subgroup the manifested clinical complications were difficulties in the natural conception, early miscarriage (9 week of gestation), recurrent miscarriages with empty gestation sac and/or stopped cardiac pulsation in the fetus.

According to the literature, other bacterial pathogens, identified with the highest rate of miscarriages, secondary infertility, infectious arthritis, endocarditis, chorioamnionitis and premature birth are *Neisseria gonorrhoeae* and microorganisms, such as *Mycoplasmas, Ureaplasmas* and *Gardnerella vaginalis* which cause bacterial vaginosis [3,4]. Our results show that *M. hominis*, *M. genitalium* and *U. urealyticum* were not detected in our tested group, which does not sustain the published data [4].

Our work reveals that 29.1% of the tested probands have positive menstrual blood results for *Ureaplasma parvum* (58.33% of all positive bacterial samples) and 27.08% *Gardnerella vaginalis* (54.17% of all positive bacterial samples). Moreover, we found high frequency (12.5%) of *UP/GV* co-infection condition. At the same time some of *GV, UP* positive patients do not have physiological obstruction or complications of the genital tract. In the last group we assume that most probably the positive menstrual blood results and the infertility are caused by infected spermatozoon during the acts of copulation. Our results confirm the hypothesis for bacterial invasion by a mechanism of vertical transmission, coming from the infected semen factor, which is in accordance with the published data [5,6]. There are lots of evidences that *Ureaplasma parvum* is a pathogen that could affect pregnancy outcome and neonatal health, especially for postmortem endometritis with septicemia and chorioamnionitis [7], as it is observed in our sample group. Our results sustain other clinical studies which proved that *Ureaplasma parvum* in the placenta and endometrium is associated with infection, stillbirth, spontaneous miscarriages, premature delivery and lowers the normal weight of newborns. Our finding that high proportion of the *UP* positive infertile women had a history of recurrent spontaneous miscarriages correlates with the published data, according to which *U. parvum* can be isolated more frequently from patients with a history of recurrent miscarriages than from normal pregnant women [8].

There is not enough data in the literature about the presence of *Ureaplasma parvum* in the menstrual blood, despite of its proven impact in the reproductive problems. For that reason, the results of our pilot study will help to clarify the causes of reproductive complications in Bulgarian women, showing infectious etiology.

As we already mentioned 27.08% of the tested probands have positive menstrual blood results for *GV* (54.17% of all positive bacterial samples). Other authors also report endometrial colonization with *Gardnerella vaginalis* and no positive infection with *Chlamydia trachomatis* or *Neisseria gonorrhoeae* [9]. The high frequency of *Gardnerella vaginalis* in our menstrual blood samples confirms the hypothesis for possible linkage between proinflammatory and inflammatory endometrial condition and unfavourable pregnancy outcome, considering the clinical data of the positive *GV* patients (predominantly early spontaneous abortions, stillbirth and biochemical conceptions) (see Table 1).

Our findings of probands who had negative cervical-vaginal swab results for *UP, GV*, or *UP/GV* co-infection, but positive results for those pathogens in menstrual blood samples, confirm undoubtedly the tropism of the described microbial pathogens to the upper genital tract and explain the detection of bacterial co-infection. Another hypothesis about the vertical transmission is by the infected male sperm factor as well. Women with bacterial vaginosis have a 50% risk for the presence of structured polymicrobial *Gardnerella vaginalis* biofilm to the endometrium and fallopian tube specimens, which biofilm can persist for a long period of time without causing symptoms in vagina and endometrium [10]. The colonization could implicate the pathogenesis of adverse pregnancy outcome in association with bacterial vaginosis, but there is an urgent need of further investigations on the microbial status in the upper genital tract.

Our results suggest that microbial invasion in the endometrium may develop a pro-inflammatory response (Th-1 bias) resulting in damage of the conception, implantation failure, spontaneous abortion, and preterm delivery, as it has already been reported [11]. A majority of evidences support the implication of bacterial products, as: endotoxin, macrophages, and other products of the Th-1 response (IL-1, TNF, etc.) in the mechanisms responsible for infection/inflammation-associated preterm delivery and fetal injury [12].

From the viral pathogens *HSV1, HSV2, CMV, VZV*, and *HHV-8* were not found in our group of tested menstrual blood samples, which contradicts to the published data [13].

EBV was detected in significantly high percentage (38.9%) of our positive viral cases, which is consistent with the published data [14]. *HHV-6* and *HHV-7* infections were detected in 11.1% and 55.5%, respectively, of all positive viral menstrual blood samples. The most common registered co-infection variants were the following: *HHV-7/GV* and *HHV7/EBV/UP* or *HHV7/UP/GV*. There is controversial data for the impact of *HHV-7* (in contrast to categorically proven

Table 1

Clinico-pathological data and infectious molecular results in 48 infertile Bulgarian women. MB-Menstrual blood

Samples	Reproductive anamnesis	Molecular results for infectious status	Infectious diseases (case history)	Genetic results	Accompanying diseases or treatment
MB-1	• difficulties in the natural conception	HHV 7	past cystitis	–	Hashimoto's disease
MB-2	• difficulties in the natural conception: 3 years • 1 miscarriage: 9 week of gestation	NEGATIVE (-)	none	–	Hormonal ovarian stimulation
MB-3	• recurrent early miscarriages	EBV	none		Hashimoto's disease; L-tyroxin therapy
MB-4	• difficulties in the natural conception • 1 miscarriage	UP	past cystitis and sexual partner with positive GV and UP infection	–	Hashimoto's disease; L-tyroxin therapy
MB-5	• 2 miscarriages: 9 and 27 week of gestation	NEGATIVE (-)	recurrent cystitis	–	–
MB-6	• difficulties in the natural conception	UP	none	–	–
MB-7	• difficulties in the natural conception: 3 years • 3 miscarriages: 6/8/9 week of gestation	NEGATIVE (-)	Several cystitis and inflammatory diseases; elevated IgM Abs for CMV and Rubeola	Trisomy 16 in the second abortive material	–
MB-8	• 2 miscarriages	HHV7; GV	recurrent cystitis; bacterial vaginosis; HSV2 and systematic fungal infection		Graves' disease; elevated MAT and Prolactin
MB-9	• difficulties in the natural conception: 3 years • 2 miscarriages (biochemical conceptions)	GV	–	–	–

Table 1

Continued

Samples	Reproductive anamnesis	Molecular results for infectious status	Infectious diseases (case history)	Genetic results	Accompanying diseases or treatment
MB-10	• recurrent miscarriages in 5-6 week of gestation	NEGATIVE (-)			
MB-11	• difficulties in the natural conception: 5 years • 1 miscarriage	NEGATIVE (-)	past infections with *Candida albicans*	–	allergies; celiac disease
MB-12	• difficulties in the natural conception	UP; GV	–	–	–
MB-13	• difficulties in the natural conception • unsuccessful IVF	NEGATIVE (-)	–	–	Raynaud's syndrome; Exposure to radiation
MB-14	• recurrent miscarriages	EBV	–	–	–
MB-15	• 3 miscarriages	NEGATIVE (-)	–	–	Hashimoto's disease; L-tyroxin therapy
MB-16	• difficulties in the natural conception	EBV; UP	–	–	Removed left fallopian tube
MB-17	• recurrent miscarriages	NEGATIVE (-)	–	–	–
MB-18	• difficulties in the natural conception: 5 years • 2 miscarriages (biochemical conceptions)	HHV 6; GV			Hypothyroidism: decreased levels of TSH and FT4; Operation of thyroid gland
MB-19	• difficulties in the natural conception	GV	–	–	–
MB-20	• difficulties in the natural conception	UP			Decreased levels of Progesterone
MB-21	• difficulties in the natural conception	UP	–	–	–

Table 1

Continued

Samples	Reproductive anamnesis	Molecular results for infectious status	Infectious diseases (case history)	Genetic results	Accompanying diseases or treatment
MB-22	• difficulties in the natural conception	UP	–	–	–
MB-23	• difficulties in the natural conception • 3 miscarriages in 7/11/18 week of gestation	NEGATIVE (-)		PAI 4G/4G; ACE I/D	
MB-24	• difficulties in the natural conception • 3 miscarriages in 5/6/10 week of gestation	NEGATIVE (-)	none	Normal female karyotype (46, XX)	–
MB-25	• difficulties in the natural conception	UP; GV	–	–	–
MB-26	• difficulties in the natural conception	UP	–	–	–
MB-27	• difficulties in the natural conception: 5 years • 5 miscarriages • 3 unsuccessful IVFs	EBV; HHV 7; UP	Infection with *Ureaplasma parvum*	46, XX, inv (20) (p12q 13.1) In embryos: monosomy: 11/ 5/ 10 Trisomy: 9/13	–
MB-28	• difficulties in the natural conception	NEGATIVE (-)	Past infections with *Candida albicans* and *Ureaplasma spp.*	Normal female karyotype (46, XX)	Hypothyroidism
MB-29	• difficulties in the natural conception • 1 miscarriage in 9 week of gestation • 1 stillbirth in 23 week of gestation	HHV 7	*Vaginal candidiasis* Elevated Ig G Abs to: VZV/EBV/CMV/ HSV1	PAI 4G/4G; ACE I/D; MTHFR (677)	

Table 1

Continued

Samples	Reproductive anamnesis	Molecular results for infectious status	Infectious diseases (case history)	Genetic results	Accompanying diseases or treatment
MB-30	• difficulties in the natural conception • 1 miscarriage in 8 week of gestation • 1 unsuccessful IVF	NEGATIVE (-)	Past infection with *Chlamydia trachomatis*	Normal female karyotype (46, XX)	–
MB-31	• 1 miscarriage in 9 week of gestation	UP	none	–	Asherman's syndrome; Metabolic syndrome; Removed fallopian tubes; Hormonal stimulation
MB-32	• 1 miscarriage in 8 week of gestation	HHV 7	None	–	Hashimoto's disease
MB-33	• difficulties in the natural conception 2 years • 2 miscarriages in 5 and 19 week of gestation • 2 natural conceptions with empty gestational sac	EBV	Past urogenital infections	PAI 4G/5G; Homozygous ACE D/D	Elevated Ig M Abs to Cardiolipin
MB-34	• 1 miscarriage in 6 week of gestation	EBV	none	–	–
MB-35	• difficulties in the natural conception 2 years • 1 miscarriage in 7/8 week of gestation	EBV	–	–	Hashimoto's disease; L-tyroxin therapy; Performed hysteroscopy

Table 1

Continued

Samples	Reproductive anamnesis	Molecular results for infectious status	Infectious diseases (case history)	Genetic results	Accompanying diseases or treatment
MB-36	• 4 miscarriages (ectopic pregnancy; empty gestational sac; stopped cardiac pulsation)	NEGATIVE (-)	Past infections with UP, *E. coli*, Vaginal *lactobacillosis*	Homozygous carrier of normal allele for PAI, FVL, MTHFR, 46, XX, 9qh+ 46, XY, 9ph+ in the sexual partner	Hashimoto's disease; L-tyroxin therapy; Elevated Ig G antiphospholipid Abs; Cavum uteri: decidual fragments with inflammatory alterations
MB-37	• difficulties in the natural conception	NEGATIVE (-)	–	–	–
MB -38	• 2 miscarriages in 4 and 8 weeks of gestation • 1 stillbirth in 6 months of gestation	GV	recurrent cystitis	–	–
MB -39	• difficulties in the natural conception	UP			
MB-40	• recurrent miscarriages	NEGATIVE (-)	–	–	–
MB-41	• difficulties in the natural conception 3 years	NEGATIVE (-)	Past infection with *Chlamydia trachomatis*		
MB-42	• 1 miscarriage in 22 weeks of gestation	NEGATIVE (-)	Urogenital infections during the pregnancy		
MB-43	• difficulties in the natural conception	HHV7	Positive clinical history for HPV 33	–	Tubal obstruction; Cysts; Endometriosis

Table 1

Continued

Samples	Reproductive anamnesis	Molecular results for infectious status	Infectious diseases (case history)	Genetic results	Accompanying diseases or treatment
MB-44	• recurrent miscarriages	HHV6	none	Normal female karyotype (46, XX)	–
MB-45	• difficulties in the natural conception	HHV7; GV	none	–	–
MB-46	• difficulties in the natural conception • 2 miscarriages in 5 and 15 weeks of gestation	HHV7; GV; UP			
MB-47	• recurrent miscarriages	HHV7; UP	–	–	–
MB-48	• difficulties in the natural conception 2 years	HHV7	Past infection with *Chlamydia trachomatis*	–	Hashimoto's disease; L-tyroxin therapy; Elevated levels of Prolactin; TSH

associations for *HHV 6*) on the fertility loss, but in our study *HHV-7* was detected with the highest frequency from all tested *HHVs* in our sample. Our data for the potential association of active asymptomatic infection with *HHV-7* in the female endometrium has to be further investigated in detail.

Conclusion. There is a great variety of factors resulting in reproductive failure. The estimated impact of infectious factors in 64.58% of the tested samples suggests that the bacterial-viral asymptomatic active infections in the upper female genital tract plays an important independent and/or complex role in female infertility.

Furthermore, our study shows the great potential of menstrual blood, as a noninvasive sample, covering endometrium, where is the direct bacterial and viral activity and the site of the future embryo implantation. Using menstrual blood as a target sample for infectious investigations will contribute to the clarification of the complex reasons for sterility and could help clinicians in the decision for endometrial biopsy. Our experience shows that treatment of the target viral/bacterial pathogens leads finally to successful conception in couples with reproductive problems.

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