



MOLECULAR DETECTION OF HUMAN HERPES VIRUS 6 (HHV-6) IN UNEXPLAINED HUMAN INFERTILITY

METHAQ HUSSEIN ABBAS

College of Science, M.Sc Nano Biotechnology, Department of Biotechnology, Acharya
Nagarjuna University, Guntur, Andhra Pradesh, India

*Corresponding Author: methaq19933@gmail.com

Received 28th Aug. 2018; Revised 24th Sept. 2018; Accepted 30th Oct. 2018; Available online 1st Feb. 2019

<https://doi.org/10.31032/IJBPAS/2019/8.2.4632>

ABSTRACT

To decide whether HHV-6 may be a reason for infertility, a contextual analysis was led of 18 men and 10 women with unexplained essential fertility, somewhere around one past pregnancy. HHV-6 DNA was distinguished in the peripheral blood mononuclear cells (PBMC) from both infertile and fertile samples (12 and 14%, individually); Endometrial epithelial cells from 4/10 (40%) infertile women were certain for HHV-6 DNA; this viral DNA was not distinguished in endometrium of fertile women. At the point when put in culture, endometrial epithelial cells delivered viral early and late proteins, recommending the nearness of infectious virus. The outcomes propose that HHV-6 disease of the endometrium triggers an unusual NK cell and cytokine profile, which thusly prompts a uterine domain that isn't good with fertility. The outcomes should be affirmed with investigations of extra fertile and infertile women. Semen tests were gathered from 18 men who were going to the infertility facility at the Government General hospital Guntur, due to couple fertility issues. The DNA of herpes viruses is every now and again distinguished in the semen of asymptomatic fertile and infertile male patients. Additionally ponders are required to research the job of herpes viruses in male/female factor infertility.

1.0 INTRODUCTION

Infertility, characterized as the failure to consider following 1 year of unprotected intercourse, is an issue that influences nearly 15%– 20% of couples in European nations. Male and female factors exist together in around 33% of infertility

cases; 60% of cases are because of a male factor, and concentrates on the causative components of male factor infertility uncover that 30% of the patients experience the ill effects of idiopathic infertility [1]. Despite the clinical significance observations, the role of herpes viruses in male factor infertility remains obscure. The prevalence of herpes viruses varies, as it has been reported until recently in the literature, particularly for HSV-6, which ranges between 3.1% and 49.5%, depending on the viral DNA detection method used [2].

Human herpesvirus 6 (HHV-6) is a betaherpesvirus that is firmly identified with human cytomegalovirus. Salahuddin et al. (1986) [3] were the first to segregate HHV-6, utilizing fringe blood lymphocytes (PBL) got from patients with lymphoproliferative clutters. At first named human B-lymphotropic virus (HBLV), cell tropism is most noteworthy for T lymphocytes [4]. Two hereditarily unmistakable variations of the virus exist, HHV-6A and - 6B. HHV-6 is a β -herpesvirus recognized with HHV-7 and, to a smaller quantity, human cytomegalovirus (HCMV). Yaminishi et al. (1998) [5] distinguished HHV-6 as a causative operator of exanthem subitum (roseola), a typical youth exanthem. HHV-6 disease is basic in the initial 2 years of life. Albeit

clear clinical malady is rare in grown-ups, HHV-6 reactivates with immune-suppression. HHV-6 has been connected with an assortment of human illnesses including numerous sclerosis (MS), despite the fact that the centrality of these affiliations is misty. Ongoing advances in the sub-atomic science of HHV6 incorporate the identification of the cell-surface receptor for HHV6 what's more, the assurance of the hereditary groupings of HHV-6A what's more, - 6B. The revelation of the cell receptor for HHV-6, CD46, has revealed another insight into HHV-6 cell tropism. Besides, the in vitro collaborations between HHV-6 and different viruses, especially human immunodeficiency virus, and their importance for the in vivo circumstance are examined, and in addition the transactivating limits of a few HHV-6 proteins. The understanding into the clinical range of HHV-6 is as yet advancing and, aside from being perceived as a noteworthy pathogen in transplant beneficiaries (as exemplified by the rising number of forthcoming clinical examinations), its job in focal sensory system ailment has turned out to be progressively clear. At long last, we present a diagram of remedial choices for HHV-6 treatment. A significant part of the ongoing literature encompassing HHV-6 has focused on the clinical range in the immune-compromised have. Reactivation

of the beneficiary's strain, exogenous disease with the contributor's strain, or reinfection with another strain may all happen. In the bone marrow transplant populace, little arrangement of patients has been detailed with interstitial pneumonitis within the sight of HHV-6, frequently in relationship with unite versus-host disease (GVHD). Viral DNA from members of the *Herpesviridae* family, such as herpes simplex virus (HSV) -1 and -2, cytomegalovirus (CMV), Epstein-Barr virus (EBV), and human herpes virus (HHV) -6, -7, -8, has been detected in the semen of asymptomatic infertile patients [6]. In particular, HSV-2 and CMV, which are sexually transmissible, have been extensively studied and can lead to fetal and neonatal abnormalities [7].

The aim of the current study was to use PCR to determine the prevalence of herpes viruses in the semen of a randomized asymptomatic male cohort attending an infertility clinic. Furthermore, the possibility of viral infections affecting semen parameters, and thus fertility, by comparing a control group to an "abnormal" semen group was also examined.

In another attempt, to elucidate the aspect of HHV-6 biology, we analyzed the presence of HHV-6 infection in two groups of women with differing levels of fertility. Specifically, we studied the prevalence of

HHV-6A and HHV-6B infection in the uterine flushing and endometrium biopsies of a randomized group of women with primary infertility group attending an infertility clinic in Guntur and a group of fertile women.

2.0 MATERIALS AND METHODS

2.1 Clinical Samples: 18 semen samples (1ml) to verify the HHV-6 and twelve blood serum samples to test the antibodies to herpes virus gathered from 18 men who were going to the infertility center at the GGH Guntur, Andhra Pradesh, (From January 2017 to December 2017) due to couple fertility issues. Informed consent was gotten from every patient for the motivations behind the present examination. Men participating had no clinical signs of HSV infection and no obvious causes for their infertility were found. All of them were infertile due to unknown causes. Individuals with any underlying diseases such diabetes which can likely interfere with fertility potential were excluded.

Endometrial tissues were collected from ladies patients (**10 samples**) that were enlisted at affirmation for tubal patency appraisal by Hystero-sono differentiates sonography. Incorporation criteria for the examination bunch were: 28-36 years of age, normal menstrual cycle (20-32 days), BMI running somewhere in the range of 17.5 and 25.5 Kg/m². The phase of the

menstrual cycle was ordered into secretory (13 to 27days). Tissue tests gathered in HEPES-supported Dulbecco adjusted Eagle medium/Hams F-12 (DMEM/F-12).

2.2 Ethics Statement: Written consent was obtained from each patient and ethics approval was obtained from the Ethical Committee of the medical centre.

2.3 Statistical Analysis: The data collected were analyzed with SPSS software version 15. The data were expressed as mean \pm SD. The differences of variables between two groups HHV (+)ve versus HHV(-) ve were compared using the Student's t-test

2.4 Analysis of semen& Blood: Semen samples were obtained by masturbation in sterile containers after sexual abstinence of 48–72 hours. Samples were analyzed within 1 hour of collection and processed for DNA extraction within 2 hours of collection. Sperm concentrations and motility of 18 male patients were examined using computer-aided sperm analysis according to the 4th edition of the WHO Guidelines [8]. Sperm morphology was assessed by observation of eosin-thiazine-stained methanol-fixed smears of fresh ejaculate under a light microscope and evaluated according to strict Krueger criteria. Normal samples were considered to be the semen specimens that fulfilled all the criteria mentioned above. An excessive number of white blood cells alone did not render the sample abnormal. All semen

specimens that had one or more altered semen parameters were characterized as abnormal. Additional parameters such as volume, progression, pH, white blood cells (WBCs), vitality, and mixed agglutination reaction (MAR) test were also evaluated.

2.5 DNA Extraction from Semen Samples & PCR Amplification Reactions

All preparations for the PCR assays were performed in a “clean room” (no post-PCR DNA products) inside a laminar flow hood to minimize contamination. After collection, each fresh semen sample was centrifuged at 13,000 rpm for 30 minutes. The supernatant (seminal fluid) was transferred to a new eppendorf tube, while the pellet containing the spermatozoa remained in the original eppendorf tube. . In the case of PCR test of semen, firstly sample DNA was extracted using DNA purification from fluid Semen kit (QIAGEN; Cat No: 57704) according what company had advised. Both spermatozoa and the corresponding seminal fluids from all participants were examined for the presence of HHV-6 DNA by PCR using the appropriate set of primers (Table 1). The PCR products were examined by electrophoresis in a 2% or 3% agarose gel, depending on the size of the PCR product, and photographed on an ultraviolet light transilluminator. Finally, HHV 6 PCR products were subjected to direct sequencing analysis, verifying the initial

amplification results (Figure 1). PCR products were purified using the QIAquick PCR purification Kit (Qiagen Valencia, CA) and eluted with water. DNA sequences were analysed by submission to the ribosomal database project website [9] for identification.

2.6 NK Cell Purification: Peripheral and endometrial NK cells were purified from PBMC and endometrial samples, respectively, through negative magnetic cell separation system.

3.0 RESULTS AND DISCUSSION

3.1 HHV-6 in Clinical Specimens of women

In the Current clinical research 10 women with unexplained primary infertility portrayed by the absence of past pregnancies or pathological determinant, and 14 ripe women with somewhere around one past fruitful pregnancy. As revealed in Table 1, no critical contrasts were available between the two companions, aside from a slight increment in estradiol levels in infertile patients ($p = 0.05$; Student t test). High estradiol levels could show that this hormone is falsely stifling Follicle-animating hormone (FSH) levels and there could be richness issues. No distinctions in progesterone levels were found. Every single clinical example were broke down for the nearness of HHV-6 disease and the outcomes are accounted for in Table 2. HHV-6 DNA was identified in 1 PBMC of

the subjects. Shockingly, the 44% of the endometrial epithelial cells from women with primary infertility were certain for HHV-6 DNA, while the accomplice of control women did not present HHV-6 viral DNA in their endometrial epithelial cells ($p = 1.27 \times 10^{-4}$; Fisher correct test). Likewise stromal cells acquired from the purging of epithelial cells were examined for HHV-6 nearness and, strikingly, no HHV-6 DNA was identified (Table 2), supporting a limited site of disease for HHV-6A into endometrial epithelium of primary unexplained infertile women. Because of the high predominance of HHV-6 dynamic disease in a level of infertile women, hunt down the nearness of contrasts in clinical and immunological parameters, subdividing infertile women based on the nearness/absence of HHV-6 contamination (Tables 3 and 4). Estradiol levels are higher in infertile women with HHV-6 disease in correlation with those HHV-6 negative ($p = 0.045$) (Table 3). The past recognizable proof of a connection among's estrogen and herpes simplex virus (HSV) reactivation from latency [10] proposes conceivable ramifications of estradiol abnormal states in HHV-6 contamination. Truth be told, we watched a relationship between's the dimensions of estradiol and the nearness of HHV-6 disease ($p = 0.001$; $R^2: 0.89$; Spearman connection). The other clinical parameters did not present contrasts in the

two accomplices (Table 3). When we thought about the immunological qualities, we watched a distinction in endometrial (e)NK cell safe phenotype and cytokine levels in the uterine condition. We found a lower CD56posCD16neg eNK cell number in HHV-6 positive infertile women (8.06) in examination with HHV-6 negative infertile women (23.82) ($p = 0.001$) (Table 4). The dissemination of CD56bright and CD56dim eNK cells was distinctive between HHV-6A positive and negative infertile women, with lower CD56bright and CD56dim eNK cells number in HHV-6 positive infertile women (3.72) in examination with HHV-6 negative infertile women (13.68) ($p = 0.001$) (Table 4). CD3+ lymphocytes were not recognized in the two associates, while CD14+ monocytes exhibited no distinctions in cell number ($p = 0.612$). The uterine flushing dimensions of cytokines demonstrated an alternate example in HHV-6 positive and negative infertile women.

Viral infections have been considered as possible environmental factors in human infertility [11]. In particular, herpesviruses have been implicated in male infertility [12], but no specific virus has yet been conclusively identified as associated with female infertility. In our report, 41% of endometrial epithelial cells from women with unexplained infertility were found

positive for HHV-6 DNA, whereas no control women (with at least one previous successful pregnancy) harbored the virus. Furthermore, endometrial epithelial cells from women with unexplained infertility harbored significant viral loads (approximately 4 copies of viral DNA per cell), but no HHV-6A infection was detected in stromal cells and PBMC, excluding the presence of chromosomally integrated HHV-6 DNA [13].

Table 5 shows the results for the prevalence of herpes virus in normal and abnormal semen samples. 9 of 18 semen samples were classified as normal (normozoospermia) according to the 4th edition WHO guidelines. Viral DNA of HHV6 was detected in 6 (33.3%) cases of normal semen samples (Table 3). Viral DNA was also detected in the abnormal zoospermia group from 18 male subjects (Table 5). In this group, the samples were further divided into the following subgroups: 6 oligozoospermic, 5 asthenozoospermic, 3 oligoasthenozoospermic, and 4 teratozoospermic. Viral DNA of HHV6 was detected 4 (22.22%), 8 (44.44%), 2 (11.11%), 3 (16.67%) and 3 (16.67%) in case of abnormal semen samples (Table 5). Statistical analysis was performed, but there was no significant statistical difference between the presence of each herpes virus and each abnormal subgroup. Viral DNA, in general, was detected by

PCR in 16 (88.88%) of 18 total semen samples for at least one member of the herpes virus family (Table 5). The results from the detection of viral DNA for all 18 samples available are HHV-6 in 16 (88.88%). All previous studies examined the prevalence of viral DNA in total semen samples. We found the possibility of detecting viral DNA in spermatozoa, in seminal fluid, or in both very intriguing, and, therefore, we separated these two biological materials by high-speed centrifugation. Interestingly, herpes virus DNA was detected at variable frequencies for each virus tested, when spermatozoa and seminal fluid were compared (Table 5). In spermatozoa, HHV-6 in 8 (44.44%), it was also combined those samples, which showed herpes virus presence both in spermatozoa and seminal fluid, constituting male semen it was found that HHV-6 in 7 (38.88%).

Table 6 shows the mean sperm count and motility of virally infected and noninfected semen samples. The mean sperm count of HHV-6 infection did not show any influence on mean sperm count. Kapranos et al. found that HHV1 infection was significantly related to low sperm count and poor motility [14]. No association was found between HHV-6+ and HSV-6- DNA and low sperm count and motility and infertility as in previous studies [15].

Normal Semen versus Abnormal Semen

Statistical analysis failed to reveal any association between the influence of herpes virus presence on the semen parameters and the subsequent characterization of samples as normal or abnormal semen, and, thus, viral existence does not seem to dramatically influence qualitative or quantitative characteristics of the semen. Viral DNA was almost equally distributed in both normal and abnormal semen. On the other hand HHV-6 appeared to be more frequent in oligozoospermic semen than in asthenozoospermic semen but with no statistical significance.

PCR results and gene sequencing

Gel analysis of PCR products showed 6 bands in PCR amplicon for β 2-globin gene fragmentation at 315 bp in all three type of sample including male and female (Figure 1), and corresponding primer sequence given in table 7. In the figure 1 right side, PCR amplification for U38 gene fragments shows 7 bands with positive control bands obtained at 467 bp. Approximate sized DNA bands were also obtained following PCR form of HHV-6 virus isolates from male (two types) and female samples. Among the two gene targets all viral isolates were positively responded to β 2-globin (315 bp) & U38 (467 bp) genes. β 2-globin is moderately

reflected in the current multi PCR technique.

Table 1: Women cohorts -demographical and clinical parameters (Average values)

Parameters	Infertile (10)	Control (14)	p value*
Age (yrs)	33.4	33.8	0.93
Duration of infertility (yrs)	2.8	2.1	0.63
Length of menstrual cycle (days)	4.6	4.3	0.43
Follicle-stimulating hormone (FSH) (mUI/mL) (day 3)	9.1	7	0.41
Luteinizing hormone (LH) (mUI/mL) (day 3)	6.7	6.2	0.41
Estradiol (pg/mL) (day 3)	76.8	65.7	0.05
Thyroid-stimulating hormone (TSH) (uUI/mL)	2.4	1.9	0.44
Free thyroxine (FT4) (pg/mL)	1.8	1.1	0.85
Progesterone (pg/mL) (day 21)	11.67	13	0.53
Day (mestrual cycle) of sample collection	13.8	13	0.80

*Student t- test

Table 2: Infertile women subdivided on the basis of the presence/absence of HHV-6 infection. Demographical and clinical parameters of infertile and control women (Average)

Parameters	HHV-6 positive	HHV-6 negative	p value*	Control (14)
Age (yrs)	33.4	32.8	0.84	33.8
Duration of infertility (yrs)	2.9	2.8	0.56	2.1
Length of menstrual cycle (days)	4.8	4.6	0.38	4.3
FSH (mUI/mL) (day 3)	9.5	9.2	0.37	7.0
LH (mUI/mL) (day 3)	7.0	6.8	0.37	6.2
Estradiol (pg/mL) (day 3)	79.9	77.5	0.04	65.7
TSH (uUI/mL)	2.5	2.4	0.39	1.9
FT4 (pg/mL)	1.9	1.8	0.76	1.1
Progesterone (pg/mL) (day 21)	12.1	11.8	0.48	13.0
Day (mestrual cycle) of sample collection	13.2	12.8	0.72	13.0

*Student t- test

Table 3: HHV-6 DNA results in peripheral blood mononuclear cells (PBMC) and endometrial biopsies.

Samples (N)	Infertile (10)	Control (14)	p value*
Endometrial epithelium	4	0	1.27×10^{-4}
Endometrial stroma	0	0	-
PBMC	1	0	2.34×10^{-5}

*Student t- test

Table 4: Infertile women subdivided on the basis of the presence/absence of HHV-6 infection. Immunological parameters in endometrial samples

Immune cells	HHV-6 positive	HHV-6 negative	p value*	Control
NK CD56posCD16neg (N)	8.06	23.82	0.001	24.78
NK CD56brightCD16neg (N)	3.72	13.68	0.001	14.89
NK CD56dimCD16- (N)	4.44	10.18	0.017	9.87
CD3+ (N)	0.00	0.00	0.000	0.00
CD14+ (N)	0.46	0.52	0.612	0.48
*Student t- test				

Table 5: Herpes viral (HHV-6) prevalence in normal and abnormal semen samples including spermatozoa and seminal fluid

	HHV-6 Positive (n=18)	%

Normozoospermia	6	33.33
Abormozoospermia	4	22.22
Oligozoospermia	8	44.44
Asthenozoospermia	2	11.11
Oligoasthenozoospermia	3	16.67
Teratozoospermia	3	16.67
Oligozoo spermia	4	22.22
Leukocyto spermia	2	11.11
Asthenozoo spermia	5	27.78
Semen (sperm and seminal fluid)	14	77.78
Spermatozoa	8	44.44
Seminal fluid	7	38.89
Total virus presence	16	88.89

Table 6.: Mean sperm count and motility in virally infected and noninfected semen samples

	Number of Specimens	Sperm Count (million/ml)	P value	Motility (mean)	P value
Viral DNA Positive	9	27.67	0.71	38.53	0.67
Viral DNA Negative	12	30.24	0.67	61.47	0.61
HHV-6 Positive	16	29.67	0.91	70.14	0.5
HHV-6 Negative	2	27.47	0.14	29.86	0.37

Table 7: Sequences of type-specific Real time PCR primers used for the detection of HHV6 in clinical samples

Virus	Gene	Primers	Primer sequence (5'-3')	Product Size
HHV-6	β2-globin	Outer	GGA GAA GGT CTT CTC GGC CTC GTA GGC TTA GTA	315 bp
		Inner	GGT CGA GTC ATC TAC GGG GAC ACG GAA TTA GGC	
HHV-6	U38	Outer	ATT AAG TTG GTA GTA CTT ACG TGA TGG TTA GGA	467 bp
		Inner	GTA TTA GGA GGT TTG AAA GGT AAG TGA GGA GGT	

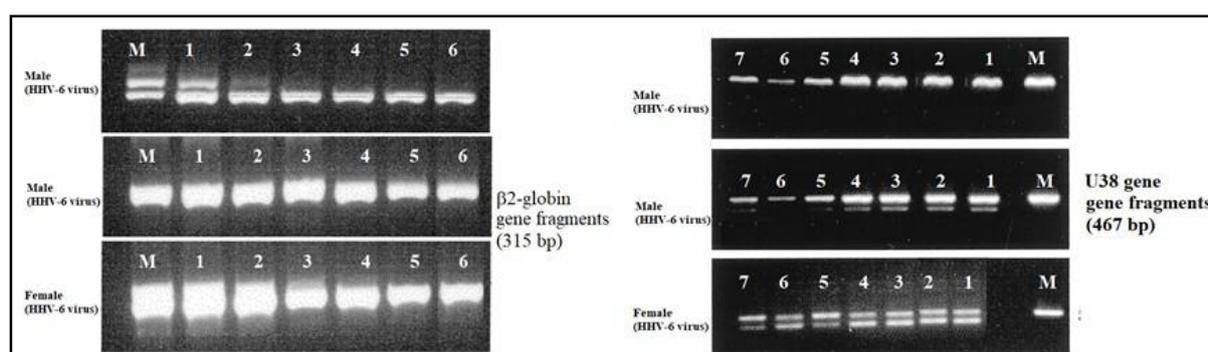


Figure 1: PCR amplification for β2-globin gene (left side) fragment (315 bp) and for U38 gene fragments (467bp) (Right side)

CONCLUSION

In conclusion, the screening of normal and abnormal semen samples for the presence of viral infection caused by any of the seven herpes viruses, for the first

time, has revealed relatively high percentages of viral infection in both groups. Whenever there is suspicion to herpes simplex as a microorganism that theoretically could impact semen

parameters and fertility potential. Overall, our study indicates that HHV-6 infection might be an important factor in male and female primary unexplained infertility.

References

- [1] Leruez-Ville M, Galimand J, Ghosn J, Briat A, Delaugerre C, Chaix ML. [Male genital tract infection: the point of view of the virologist]. *Gynecol Obstet Fertil* 33, 2005, 684–90.
- [2] Moore DE, Ashley RL, Zarutskie PW, Coombs RW, Soules MR, Corey L. Transmission of genital herpes by donor insemination. *JAMA* 261, 1989, 3441–3
- [3] Salahuddin SZ, Ablashi DV, Markham PD, et al. Isolation of a new virus HBLV, in patients with lymphoproliferative disorders. *Science* 234, 1986, 596-601.
- [4] Lusso, P., Malnati, M., De Maria, A., Balotta, C., DeRocco, S. E., Markham, P. D. & Gallo, R. C., Productive infection of CD4 β and CD8 β mature human T cell populations and clones by human herpesvirus 6. Transcriptional down-regulation of CD3. *J Immunol* 147,1991, 685–691.
- [5] Yamanishi, K., Okuno, T., Shiraki, K., Takahashi, M., Kondo, T., Asano, Y. & Kurata, T. Identification of human herpesvirus-6 as a causal agent for exanthem subitum. *Lancet* i, 1988, 1065–1067.
- [6] Bezold G, Schuster-Grusser A, Lange M, Gall H, Wolff H, Peter RU. Prevalence of human herpesvirus types 1–8 in the semen of infertility patients and correlation with semen parameters. *Fertil Steril* 76, 2001,416-418.
- [7] Dejuq-Rainsford N, Jegou B. Viruses in semen and male genital tissues—consequences for the reproductive system and therapeutic perspectives. *Curr Pharm Des* 10, 2004,557–75.
- [8] World Health Organization Laboratory Manual for the Examination of Human Semen and Sperm-cervical Mucus Interaction, 4th ed. New York: Cambridge University Press, 1999.
- [9] <http://rdp.cme.msu.edu/>
- [10] Vicetti Miguel RD, Sheridan B, Harvey SA, Schreiner RS, Hendricks RL, Cherpes TL. 17-beta estradiol promotion of herpes simplex virus type 1 reactivation is estrogen receptor dependent. *J Virol.* 84, 2010, 565–572. pmid:19846508
- [11] Baecher-Lind LE, Miller W, Wilcox AJ. Infectious disease and reproductive health: a review.

- Obstet Gynecol Surv. 65, 2010, 53–65. pmid:20040130
- [12] Naumenko V, Tyulenev Y, Kurilo L, Shileiko L, Sorokina T, Evdokimov V, et al. Detection and quantification of human herpes viruses types 4–6 in sperm samples of patients with fertility disorders and chronic inflammatory urogenital tract diseases. *Andrology*. 2, 2014, 687–694
- [13] Pellett PE, Ablashi D, Ambros PF, Agut H, Caserta MT, Descamps V, et al. Chromosomally integrated human herpesvirus 6: questions and answers. *Rev Med Virol*. 22, 2012, 144–155
- [14] Kapranos N, Petrakou E, Anastasiadou C, Kotronias D. Detection of herpes simplex virus, cytomegalovirus, and Epstein-Barr virus in the semen of men attending an infertility clinic. *Fertil Steril* 79(Suppl 3), 2003, 1566–1570
- [15] Volpi A. Severe complications of herpes zoster. *Herpes* 14(Suppl 2), 2007, 35–9.