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ORIGINAL ARTICLE

Molecular detection of HHV-1, HHV-2, HHV-5 and HBV in semen of fertile and infertile men by multiplex PCR method

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ABSTRACT

Background and Objectives: A great diversity of factors including viruses such as human herpes virus 1&2 (HHV-1&2), human herpes virus 5 (HHV-5), and hepatitis B virus (HBV) play key roles in sterility and it is worth noting that male infertility accounts for nearly 50% of barrenness, globally. In this regard, we evaluated the prevalence of the aforementioned viruses in semen specimens of two distinct groups of men referred to Novin Infertility Center in Mashhad, Iran.

Materials and Methods: In this cross-sectional study, 300 semen samples were collected from 150 infertile and 150 fertile men. Subsequently, genomic DNA was extracted before performing multiplex polymerase chain reaction (PCR). Eventually, the results were analyzed via SPSS Statistics V.16.0.

Results: Out of 300 specimens, 183 (61.1%) were positive at least for one of the forenamed viruses; genome detection of HHV-1&2, HHV-5, and HBV were 27%, 18%, 36.66%, and 4%, respectively.

Conclusion: The current study found no correlation between infertility and HBV, HHV-5, and HHV-1&2, which may have to do with factors like sample size, the geographical distribution of the viruses, and the lifestyle (sexual behavior) of the participants. These results emphasize the implementation of such studies on a broader scale to determine the exact factors involved in infertility.

Keywords: Herpesvirus 1; Human; Herpesvirus 2; Human; Cytomegalovirus; Hepatitis B virus; Infertility

INTRODUCTION

Infertility exerts calamitous influences on relatively 15% of reproductive-aged couples, and male infertility constitutes just about 50% of infertile cases around the world. Based on Maharloui et al. the infertility rate in Iran is 6% in women, 3% in couples, and 2% in men (1). Additionally, it is well worth mentioning infertility, as one of the most prominent factors accounting for social health, psychological and financial problems (2). Although a great diversity of factors including genital injuries, semen infections, testes, genitals and appendages, varicocele, genital obstruction, and endocrine and metabolic diseases

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can be counted as the causative agents of male infertility, genital tract infections have been mentioned as the most influential ones (3, 4).

Despite the discovery of antibiotics and vaccines, in addition to the improvement of disorder prevention and management programs, sexually transmitted infections (STI) have remained a paramount cause of acute and persistent diseases with probable involvement in pregnancy issues and infertility (5). Heading the list of chronic viral sperm infections includes: human immunodeficiency virus (HIV), hepatitis B (HBV), and hepatitis C (HCV) viruses, also little is known about human papilloma virus (HPV) semen infection; however, the growing evidence suggests that this virus might play a major role in male infertility. Moreover, other common sexually transmitted viruses including human herpes viruses (HHV), human cytomegalovirus (HCMV), and adeno-associated virus (AAV) with the ability of colonization in semen are the major concerns of reproductive specialists (6). Furthermore, the literature has proved noticeable associations between the presence of HHVs DNA in semen as well as such sperm anomalies as reduced sperm count, motility, and male infertility. DNA of HHV-1&2 and HHV-5 has been detected in 0 to 62.5% of semen samples. Although HHV-5 can also infect spermatozoa and reduce the rely and motility of sperm, some research failed to show a noteworthy correlation between HHV-5 infection and odd sperm parameters, at the same time others testified the degradation of sperm parameters and sperm features in HHV-5 virus infections (6-9). According to a study, HBV infection may lead to male infertility as a result of damaging sperm, dwindling sperm count, motility, deformity, and viability. HBV induces reactive oxygen species (ROS) and curtails the antioxidant capacity of sperm, resulting in oxidative stress and sperm dysfunction. Additionally, this virus can have non-specific and mutagenic effects on sperm chromosomes. As stated in studies concerning the effects of this virus, it can exert inherited effects on offspring (10, 11).

Since a soaring rate of viruses is present in semen-free fluid or such semen cells as sperm, macrophages, and lymphocytes, this fluid as one of the most essential carriers plays an outstanding role in the spreading of viral infections. What's more, not only can viral germ cell infection alter testicular function, but also it can transmit mutations to the next generation. In other words, mutations caused by viral infections can occur in sperm-producing cells and consequently, infertilization can be passed on to the next generation or cause pernicious fetal damage. It is also significant to note the fact that even if viral infections are diagnosed in the genital tract, the side effects resulting from antiviral therapies may exert negative impacts on the genital tract, demonstrating the importance of monitoring the prevalence of viral infections in the genital tract (9). In general, although some studies have shown the effects of viral infections in various semen parameters, others were not able to distinguish any noticeable relationship between these viral infections and the incidence of infertility in men. In this regard, we designed this study to determine the rate of HHV-1&2, HHV-5, and HBV in semen and the opportunity of affecting seminal parameters such as lowering sperm remember and motility, Changes in environmental pH and sperm morphology, etc. which leads to defections in fertility.

MATERIALS AND METHODS

This cross-sectional study was approved by the committee for the ethics of medical experimentation on human subjects, Golestan University of Medical Sciences, Gorgan, Iran (Ethics code: IR.Goums.REC.1395.248). Additionally, Participants have been informed by doctors about this study and written informed consent was taken from them. A total of 300 semen samples were collected from 150 infertile and 150 fertile men admitted to Novin Infertility Center in Mashhad, Iran, between September 2016 and June 2017 for fertility evaluation. Furthermore, those who had records of genital lesions had been excluded. Afterward, seminal parameters together with volume, pH, sperm count, motility, viability, and morphology had been measured according to World Health Organization (WHO) (2010).

DNA extraction and cloning. Briefly, Genomic Blood DNA (gDNA) extraction kit (GenetBio, South Korea) was used for DNA extraction from semen samples according to the kit's instruction (4). Quality of the extracted DNA was evaluated by the Nanodrop device (DeNovix, USA). Following, positive control plasmid prepared by using confirmed clinical positive HHV-1&2, HHV-5 and HBV cases and the colonization of PCR product of each virus was performed by

DNA-cloning kit (T/A vector, Thermo scientific, cat number: #k1231); and confirmed by Colony-PCR.

PCR method. Multiple set of primers designed for, UL5 of HHV-2, UL27 of HHV-1, UL54 of HHV-5 and C antigen of HBV using MPPrimer software. Designed primers were as followed:

UL5 (412 bp); F 5'-CGCGCCTCCGAAAGATGGTGTT-3' / R 5'-TCGTCCAGCCCGGCGAAGATAA-3',

UL27 (217 bp); F 5'-GACGTCACCGTTTCGCAGGTGT-3' / R 5'-CGTTGGCCGGTTTCAGCTCCAT-3',

UL54 (571 bp); F 5'-CACACCGCTTTGTGCGTGCTTC-3' / R 5'-AACTCCAGCTTGACGGGCTCCA-3',

AgC (310 bp); F 5'-CGCAGCAGGTCTGGAGCAAACA-3' / R 5'-ATGCGACGTGCAGAGGTGAAGC-3'.

PCR amplification were performed in a final volume of 25 µL containing 100 ng of DNA template, 0.4 mM deoxynucleotide triphosphate (dNTP) mix, 1× PCR buffer (Amplicon™, Denmark), 3 mM $MgCl_2$, 0.4 pm primer mix, 2.5 U Hot start Taq DNA polymerase (Amplicon™, Denmark), 1 M Bettaine. The cycling conditions consisted of an initial denaturation at 95°C for 2 min, 45 cycles of melting at 95°C for 1 min, annealing at 61°C for 45 s, and elongation at 72°C for 1 min and a final extension at 72°C for 10 min. To verify the presence of the amplification products, 10 µL of each PCR was separated by electrophoresis on a 1.5% agarose gel. A 100-bp DNA molecular marker was included in some of the electrophoresis runs (Sinacolon™, Iran) (Fig. 1).

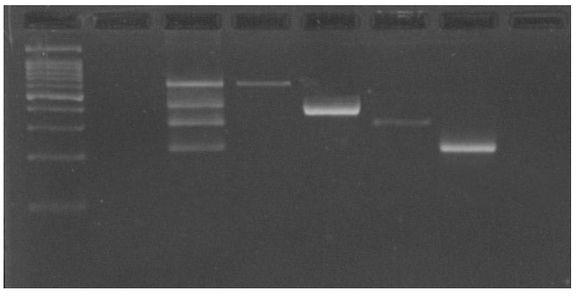

Fig. 1. Gel electrophoresis of the amplicons obtained from the multiplex PCR primer designed. The first line is DNA ladder 100 bp. The second line is the negative control. The third line is the mixture of the positive control samples. The fourth line is the CMV positive sample, the fifth line is the HHV-2 positive sample, the sixth line is the HBV positive sample and the seventh line is a HHV-1 positive sample.

In order to check the LOD (Limit of Detection) of the designed PCR protocol, the control plasmids were mixed in an equal proportion (10^6 plasmids from each control per microliter) in a vial and a 10-fold serial dilution of it was prepared. After performing PCR on these samples, the PCR products were run on gel electrophoresis and the last serial dilution, in which all controls were visible was determined as LOD. After repeating the experiment 3 times, the results showed that the LOD of this study was 10^3 viral particles per microliter.

The quality of all extracted DNA samples was assessed by Beta-actin gene PCR primers. All internal control positive samples were subjected to Multiplex PCR set. Moreover, negative and positive controls were included in all PCR reactions. Clinical positive samples from the previous studies were utilized as positive controls and double distilled water (DDW) was used as a negative control.

Statistical analysis. The results of current study were analyzed via SPSS Statistics V.16.0 (SPSS Inc., Chicago, IL, USA). Qualitative variables were tested using the Chi-Square test. P values ≤0.05 were considered statistically significant.

RESULTS

A total of 300 semen samples were collected from fertile and infertile men over a 9-month period. The participants ranged from 23-67 years with an average of 33.1 years old (see Table 1). The mean spermia was 3 ± 1.4 mL in the fertile group and 2.7 ± 1.6 mL in the infertile group (p = 0.59; p = 0.001), so a significant difference (< 1.5 mL) was observed between the two studied groups (p = 0.006). Moreover, the mean sperm count was considerably lower in the infertile subjects compared with the fertile ones (12.6 ± 15.2 vs. 54.83 ± 21.9 million/mL, respectively; p = 0.001). Also, the samples with a sperm count < 15 million/mL were significantly more frequent in the infertile group in comparison with the fertile one (p = 0.001). Furthermore, sperm motility in the fertile participants was 50 ± 8.4 which was dramatically higher than infertile individuals (26.7 ± 12.8, p = 0.001). What's more, sperm motility < 32% and sperm morphology < 4% were significantly higher in the semen of infertile men compared with the fertile ones (p = 0.001).

Based on molecular analyses, out of 300 samples,

Table 1. Cumulative results of seminal criteria of samples

	MEAN ± SD	MAX	MIN
AGE	33.1 ± 4.9	67	23
SEMEN VOLUME (ML)	3.01 ± 1.69	10.5	0.9
SPERM MOTILITY (%)	47.72 ± 21.84	81.31	0.0
SPERM COUNT (MILLION)	221.46 ± 276.78	2352	0.0
SPERM VITALITY (%)	54.38 ± 23.01	86.37	0.0
SEMEN PH	7.644 ± 0.344	8.5	6
NORMAL MORPHOLOGY (%)	16.07 ± 15.12	51.3	0.0

183 (61.1%) were positive at least for one of the aforementioned viruses (HHV-1&2, HHV-5, and HBV). The genome detection of HHV-1&2, HHV-5, and HBV were 27%, 18%, 36.66% and 4%, respectively. It is worth considering that among 183 positive samples, 38 (20.7% of all samples) were positive for two, 18 (9.83% of all samples) for three, and 12 (6.5% of all samples) for four viruses, simultaneously. Our molecular outcomes confirmed that 10 (6.6%) out of the 150 samples of infertile men and 2 (1.3%) out of 150 samples of fertile ones had been superb for HBV, which used to be statistically insignificant (p = 0.06). The detected co-infections had been noted in Tables 2 and 3.

DISCUSSION

Infertility has emerged as a prevailing health issue and male elements are accountable for up to 50% of the cases. Although the motives of male infertility are still obscure, it looks that urogenital infections are accountable for infertility in 6-10% of instances (11). According to the literature, viral infections including HHV-1&2, HHV-5, and HBV can infect semen, and exert negative impacts on sperm characteristics and semen quality which eventually lead to infertility (6, 9).

In this study, the overall rate of HHV-1&2, HHV-5 and HBV was 61.1% with a similar prevalence of the four mentioned viruses in distinct age groups. There are some controversial reports regarding the prevalence of HHV1-2 in semen in diverse studies. A review article published in 2013 showed the frequencies of much less than 4% in both fertile and infertile men in more than 1/2 of the studies. However, some research located HHV-1&2 in nearly half of the semen samples got from fertility clinics (12). Neofytou et al. referred that the presence of HHV DNA in semen was related to a reduction in sperm awareness and motility (13), however; this was not confirmed by Kaspersen. Also, a significant correlation between HHV infection and a lower spermia as well as a lower mean sperm count was reported (12).

Table 2. Comparison of molecular detection of HHV-1&2, HHV-5 and HBV and seminal criteria of samples

VIRAL DNA	N (%)	MEAN SPERM COUNT (MILLION/ML)	MEAN SPERM MOTILITY (%)	SEMEN VOLUME (ML)	SPERM VITALITY (%)	NORMAL MORPHOLOGY (%)
HHV-1+	81 (27%)	19.8*	39.1	2.5*	53.3	14.7
HHV-2+	54 (18%)	21.7*	45.3	3.7	54.7	13.8
HHV-5+	110 (36.66%)	46.9	47.7	2.8*	56.6	6.33**
HBV+	12 (4%)	34.5	42.3	4.9	66.3	4.33**
MULTIPLE INFECTION OF 2 VIRUSES	38 (20.7%)	10.4**	35.3*	2.7	45.2	8.43**
MULTIPLE INFECTION OF 3 VIRUSES	18 (9.83%)	11.8**	33.4**	1.3**	32.1**	3.21**
MULTIPLE INFECTION OF 4 VIRUSES	12 (6.5%)	4.5**	33.7**	1.6**	23.7**	1.1**

*= significant at 0.05 level

**= significant at 0.01 level

***= significant at 0.001 level

Table 3. Comparison of viral DNA detection positive samples and seminal criteria of samples

	POSITIVE	NEGATIVE	CHI SQUARE TEST P VALUE
SEMEN VOLUME	3.71 ± 1.49	4.25 ± 1.81	0.057
SEMEN PH	7.61 ± 0.32	7.66 ± 0.35	0.42
SPERM COUNT	218.93 ± 241.77	223.57 ± 304.49	0.92
SPERM MOTILITY	48.88 ± 20.08	46.75 ± 23.29	0.561
SPERM MORPHOLOGY	16.23 ± 16.14	15.94 ± 14.32	0.91
SPERM VITALITY	55.88 ± 20.50	53.13 ± 24.98	0.467
DATA SHOWN IS MEAN ± SD.			

Based on Monavari et al. 20 and 15% of infertile participants had been infected with HHV-1 and 2, respectively. Moreover, 56.6% of semen samples of infertile folks were contaminated with HHV-1 (14). They also confirmed that the semen samples infected with HHV-1 had considerably lower sperm motility than non-infected ones. Moreover, HHV was present in 3.7% of the semen samples of infertile men, and HHV presence was once associated with a limit in sperm depend and motility. Furthermore, in a review by Ochsendorf et al. a noteworthy relationship was determined between the presence of HHV and infertility that was in distinction with the result of current study (5). Additionally, no significant association was observed between the prevalence of HHV-1&2 and infertility (P=0.06) in this study; however, the correlation between spermia (P<0.05) and sperm count (p<0.05) with the presence of the viruses was significant.

In the current investigation, HHV-5 DNA was detected in 42% of infertile and 31.33% of fertile men, respectively. Although the prevalence of HHV-5 was not significantly associated with infertility (P=0.3), the correlation between sperm morphology and spermia in the studied specimen and the presence of the virus in these samples was meaningful (P<0.001). Naumenko et al. discovered that HHV-5 might also disturb sperm maturation through direct toxic effects (15). Nevertheless, other studies failed to show the same associations (15, 16). A cross-sectional study by Kapranos et al. detected HHV-5 DNA in 7.1% of semen samples (17). However, no association has been found between HHV-5 infection and sperm characteristics. In 2019, an investigation on one hundred seventy infertile men by Eggert-Kruse et al. declared that the presence of HHV-5 in semen does not notably have an effect on the superb of sperm (18). Besides, in a case-control study on semen samples in Iran, HHV-5 DNA was detected in 6% of infertile and 4% of fertile men, respectively (19). This is similar to the current study which showed no association between semen parameter abnormality and HHV-5 seminal contamination.

Research on transgenic mice elucidates the viable hyperlink between HHV and infertility. The implication of HHV in male infertility and the facility of its detection the usage of Multiplex PCR method paved the way for distinguished therapeutic interventions, as validated through preliminary trial of three men that lead to profitable pregnancies after antiviral therapy (20). Also in all likelihood influences of HHV contamination in the failed cases of fertilizations using IVF strategies proved the magnitude of these viruses in the IVF method via dint of low-quality semen derived from infertile males. Moreover, HHV-5 DNA was once detected in 5.1% of cryopreserved semen samples, ensuing in serious risks for transmission to fetus and its developmental abnormalities due to the removing of natural spermatozoa selection (17). Consequently, the molecular analysis of semen specimens regarding the presence of herpes viruses, in advance of any intervention using assisted reproduction techniques would be considerably beneficial.

One investigation in 2021 proved that sperm motility and ordinary sperm morphology have been drastically negatively affected in HBV-positive men (21). Our results confirmed that 6.6% and 1.3% samples of infertile and fertile men have been positive for HBV, respectively, which was not statistically significant. Although here, more positive HBV semen samples were detected in infertile participants compared with those of fertile group, the difference was not statistically significant. However, in the present study, the correlation between sperm morphology and viral DNA was significant (P<0.001). The result of this study was in line with that of Zangeneh and colleagues stating that the frequency of HBV in in-

fertile persons was considerably low and not statistically different from that of fertile men (22). Additionally, a study in Ahvaz indicated a very low frequency of HBsAg among infertile couples, subscribed to the results of this investigation. Moreover, it has been reported that none of the semen samples in infertile men were tremendous for HBV DNA, which could be explained by the low prevalence of HBV infection in their population (23). In contrast, a case-control study comparing men with HBV infection with those without HBV exhibited an increased risk of infertility (24). Also, a systematic review promotes the idea stating that HBV infection could probably be responsible for male infertility (24). Therefore, in accordance to the above-mentioned studies, it seems that the low prevalence of HBV infection in infertile persons, as well as the small sample size of our study, can also influence the HBV affiliation with male infertility.

According to the knowledge of the authors, no study was found on the effect of viral co-infection on sperm parameters, but the current study documented the co-infection between HHV-1 and HHV-5 as the highest percentage of co-infection assenting the hypothesis claiming a remarkable correlation between co-infection with two viruses and sperm count, motility and morphology. In addition, co-infection with 3 and 4 viruses indicated a significant relationship between co-infection of these viruses and abnormality in all sperm parameters, which manifests the importance of co-infections, especially the viral ones, in the male reproductive system and their effects on fertility. Besides, simultaneous infection with several viruses can exacerbate the complications of each virus, or by synergic affecting the count, motility, and morphology of sperm, these infections can dramatically reduce a person's fertility.

CONCLUSION

In conclusion, although there was an association between semen quality and the detection of HHV-1&2 and HHV-5, and HBV in semen samples, no significant correlation has been observed between the viruses and male infertility. Altogether, despite several studies which exhibited associations between infertility and HBV, HHV-5, and HHV-1&2, others do not assent to these findings. This flagrant discrepancy may be traced to various factors including the differences in sample size, the geographical distribution of the viruses, and the lifestyle (sexual behavior) of the participants. Also, it has to be referred to that the prevalence of one-of-a-kind viruses in semen, and their relevance to male infertility, differs drastically due to the genome extraction and amplification techniques or due to a real variation between study populations and geographical regions. Among the limitations of this study, we can point out the lack of using more sensitive techniques such as Real-Time PCR and the lack of separation of the sperm from the liquid in semen and the separate study of both of them. Moreover, such technical issues as the sensitivity of detecting methods (PCR or real-time PCR) as well as differences in the extraction procedures may also clarify mismatches between diverse literature studies.

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