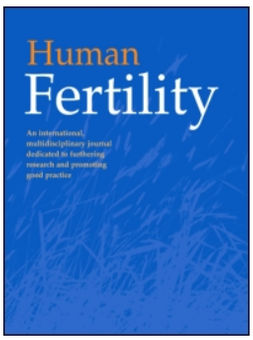

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ORIGINAL ARTICLE

Molecular detection of HHV1-5, AAV and HPV in semen specimens and their impact on male fertility

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ABSTRACT

Viral infections have been considered as possible destructive factors that influence male fertility. The aim of this study was to determine the prevalence of human herpes viruses 1-5 (HHV1-5), adeno associated virus (AAV) and human papilloma virus (HPV) in semen and whether these influence semen quality. DNA extraction was performed using phenol–chloroform protocol, then three different nested-PCRs were done to detect HHV1-5, AAV and HPV DNAs in the semen samples. Of 145 samples, 66 (45.5%) were positive at least for one of the viruses. The genome detection rate of HSV1/2, VZV, EBV, HCMV, AAV and HPV were zero, 2.8%, zero, 1.4%, 27.6% and 19.3%, respectively. Of 66 positive samples for these viruses, 6 (4.1% of all samples) were positive for two viruses simultaneously. Here no association was found between variations in semen parameters related to fertility and detection of VZV, HCMV, AAV and HPV DNA in semen samples. It should be noted that the prevalence of different viruses in semen, and their relevance to male infertility, differs significantly due to the genome extraction and amplification methods or due to a real variation between study populations and geographical regions.

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Introduction

Infertility affects around 15% of couples (Neofytou, Sourvinos, Asmarianaki, Spandidos, & Makrigiannakis, 2009) in which male infertility contributes to 20–50% of all cases (Chen et al., 2013). A wide variety of elements can cause infertility in men (Kapranos, Petrakou, Anastasiadou, & Kotronias, 2003; Naumenko et al., 2014; Oliva, Spira & Multigner, 2001). Bacterial and viral infections have been considered as possible destructive factors that influence male fertility. Both direct and indirect effects of human viral infections can disturb reproductive procedures (Garolla et al., 2013), including spermatogenesis, the function of sperm and its transport (Monavari et al., 2013). Direct toxic effects of viral infections on cells of the male genital tract or indirectly by immunological reactions that adversely affect reproductive function may contribute to male fertility disorders (Neofytou et al., 2009). In general, male fertility depends on the quantity and quality of sperm. Different kinds of sperm anomalies such as abnormal count, morphology and motility have been found to correlate with viral infections (Schlehofer, 2003). Even in asymptomatic men, semen infections are frequently present and are often associated with poor semen quality (Bezold et al., 2007; Garolla et al., 2013).

The role of some human herpes viruses (HHVs) from *Herpesviridae* family, in male infertility has been investigated in various studies (Chen et al., 2013; Naumenko et al., 2014; Neofytou et al., 2009) and the association between detection of their DNA in semen and sperm anomalies such as reduced sperm count and motility has been shown (Bezold et al., 2007; Garolla et al., 2013; Kapranos et al., 2003).

Herpes simplex virus (HSV) is a common virus in human population (Monavari et al., 2013). The DNA of both HSV-1 and HSV-2 has been detected in 2 to 50% of semen samples (Garolla et al., 2013; Kapranos et al., 2003; Monavari et al., 2013; Neofytou et al., 2009). In the initial analysis by Bezold et al. (2007) on the presence of HHV1-8 in German men seeking fertility evaluation, one or more herpes viruses have been found in 18.7% of semen samples. Notably, in the studies from Greece much higher frequencies of HHVs were reported in either normal semen samples or semen from men attending fertility clinics (Kapranos

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et al., 2003; Neofytou et al., 2009). In the Kapranos et al. (2003) study, approximately half of the specimens were positive for HSV. Some studies reported the association between HSV infection and diminished sperm quality, whereas the others reported no significant correlation (Naumenko et al., 2014).

The prevalence of human cytomegalovirus (HCMV) in semen samples from different countries varies from 0 to 62.5% (Kaspersen & Höllsberg, 2013). Although HCMV may infect spermatozoa and decrease the count (Kaspersen et al., 2012; Naumenko et al., 2011) and motility (Kaspersen et al., 2012) of sperm, some studies were unable to show significant correlation between HCMV infection and abnormal sperm parameters (Eggert-Kruse, Reuland, Johannsen, Strowitzki, & Schlehofer, 2009; Naumenko et al., 2014). The other member of the HHVs is Epstein Barr virus (EBV), which is the common cause of infectious mononucleosis. This virus establishes a latent infection after primary infection and causes periodic replication principally in the oral and genital mucosa (Gianella, Ginocchio, Daar, Dube & Morris, 2016). As EBV is found in semen and can be transmitted sexually, its effect on fertility was investigated in some studies (Bezold et al., 2007; Kapranos et al., 2003).

Orchitis is one of the rare complications of varicella zoster virus (VZV), another member of HHVs, which may consequently lead to testis atrophy and affect sperm production (Anyabolu, 2016).

In many studies the detection of adeno-associated virus (AAV) genome, a member of *Parvoviridae* family, in the female genital tract and also in miscarriage material has been reported (Burguete et al., 1999). Some of these studies even reported the correlation between recurrent abortions in women and the presence of AAV genome in semen of their partners (Kim et al., 2012). AAV genome especially has been found in the sperm fraction of semen. The possibility of AAV sexual transmission has been cleared and its genome frequently was found in semen of both infertile and fertile men; therefore its role in male infertility is vague (Erles et al., 2001). In addition to the host cell, AAV as a satellite virus needs a helper virus such as human papillomavirus (HPV) or HHV.

HPV is one of the most important sexually transmitted viruses both in males and females (Green, Monteiro, Bolton, Sanders, & Gibson, 1991). The role of HPV in the development of many malignancies has been demonstrated. Moreover, many documents showed the existence of HPV in testicular tissue of infertile men, so the association between HPV infection and disturbed sperm parameters is feasible (Nasseri et al., 2015). This correlation has been shown by some studies that reported reduced sperm motility and pH of seminal fluids in cases of HPV infection. Although the other studies were not able to distinguish any significant changes in this regard (Garolla et al., 2013).

The aim of the present study was to determine the prevalence of HHV1-5, AAV and HPV in semen and the possibility of affecting seminal parameters and fertility by infection with these viruses.

Materials and methods

Semen samples and analysis

A cross-sectional study was performed to detect the genome of HHV1-5, AAV and HPV in 145 semen samples collected from three private medical laboratories between December 2015 and May 2016 in Tehran, Iran. We included people who attended the medical laboratory seeking fertility evaluation and excluded those who had the history of genital lesions. Each sample was divided into two equal parts: one part was used for semen analysis and the other for DNA extraction. Seminal parameters including volume, pH, sperm count, motility, viability and morphology were measured according to World Health Organization (WHO) (2010). This study was approved by the ethics committee of School of Public Health, Tehran University of Medical Sciences (IR.TUMS.REC.1395.239).

DNA extraction

All samples were centrifuged at 650×*g* for 15 min. The pellets containing the sperm were treated with proteinase K to remove proteins. Then using the phenol-chloroform protocol, DNA was extracted and subjected to spectrophotometry for quantification.

PCR assays and type identification

For all samples the quality of extracted DNA was assessed by HLA gene PCR using following set of primers: HLA-F (5′-TGGTGTAAACTTGTACC-3′) and HLA-R (5′-GGTAGCAGCGGTAGAGTT-3′). All HLA-positive samples were subjected to three different sets of nested-PCRs for HHV1-5, AAV and HPV. Negative and positive controls were included in all PCR reactions. For positive controls, clinical samples from previous studies with a positive result for each virus were used and double distilled water (DDW) was used as the negative control. HPV nested PCR was performed using MY09 and MY11 primers for the first round and GP5 + and GP6 + primers for the second round (Aghakhani et al.,

2011). HHV1-5 multiplex nested PCR was implemented using two sets of primers for each round according to Kharazani Tafreshi, Ahadi, Amini-Bavil-Olyaee, & Roostaee (2004) and AAV hemi-nested PCR was done using primers from authors previous study (Shafiei-Jandaghi et al., 2017). The PCR products of samples with positive results for HPV and HHV PCR reactions were subjected to nucleotide sequencing to identify the type of virus. Finally using BLAST tool in Gene Bank, sequences were evaluated and genotypes were determined.

Statistical analysis

The statistical analyses were done using SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). Chi Square test was used to compare the seminal parameters between positive cases for detected viruses and negative ones. A p value of <0.05 was considered statistically significant.

Results

In this cross-sectional study 145 semen samples from men referred to private laboratories in Tehran were collected. The presence of HSV1/2, HCMV, VZV, AAV and HPV genomes were examined in these samples to assess their effects on male infertility through the evaluation of semen quality based on WHO (2010) criteria.

The age of participants ranged from 18 to 72 years with an average of 34.6 years (see Table 1). Since in this study the participants attended the medical laboratory seeking fertility evaluation, we did not follow the participants' final diagnoses by physician, so we cannot report the actual number of infertile people.

Of 145 samples, 66 (45.5%) were positive at least for one of the mentioned viruses. The genome detection rate of HSV1/2, VZV, EBV, HCMV, AAV and HPV were zero, 2.8%, zero, 1.4%, 27.6% and 19.3% respectively. It is worth noting that among 66 positive samples for these viruses, 6 (4.1% of all samples) were positive for two viruses simultaneously. The detected coinfections were observed as follows: two samples for AAV and HPV, one sample for AAV and HCMV, one sample for HPV and HCMV and two cases for VZV and HPV. Sequence analysis showed that among 28 positive cases for HPV, type 16 were detected in 60%, type 9 in 17%, type 39 in 7% and types 43, 45, 53 and 55 each one in 4%.

Table 1. Minimum, maximum and mean values of age and semen characteristics.

	Mean ± SD	Max	Min
Age	34.62 ± 8.9	72	18
Semen volume (ml)	4.01 ± 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

The results revealed that none of the semen characteristics (including volume and pH of the semen samples also number, viability, motility and morphology of sperm) were significantly different between the 66 positive samples for the mentioned viruses and the 79 negative samples (Table 2). In addition, a comparison of seminal features between different age groups (18–29, 30–44 and 45–72 years) showed no significant differences.

Discussion

In this study, the overall detection rate of HSV1/2, VZV, EBV, HCMV, AAV and HPV genomes in semen samples of 145 Iranian men referred to private medical laboratories in Tehran was 45.5%. Comparison of semen quality between samples with positive and negative results for DNA detection of all mentioned viruses together also for each of them separately, showed no significant differences. In addition, the detection rate of viruses' genome in different age groups was similar. By excluding people who had the history of genital lesions, we managed to reduce the risk of contamination. Meanwhile for more certainty in this regard, all samples were centrifuged and extraction was performed on the sperm pellets. These findings were similar to the results of some studies but were different from the others.

The HSV1/2 prevalence in semen varies among different studies. A review article published in 2013 showed the frequencies of less than 4% in both fertile and infertile men in more than half of the studies (Kaspersen & Höllsberg, 2013). However, some studies (Eggert-Kruse et al., 2009; Kapranos et al., 2003; Neofytou et al., 2009) found HSV1/2 in almost half of the semen samples obtained from fertility clinics. Neofytou et al. (2009) showed that the presence of HSV DNA in semen was associated with a reduction in sperm concentration and motility, although this was not confirmed by Kaspersen and Höllsberg (2013). In the present study no HSV1/2 DNA was detected in semen samples regardless of semen quality.

The presence of VZV in semen has been investigated in limited studies. Most of them indicated that VZV is not present in semen regardless of fertility (Kaspersen & Höllsberg, 2013), but in a study in Greece 1.2% of semen samples were infected with VZV (Neofytou et al., 2009). Here 2.8% of semen samples were positive for

Table 2. Comparison of seminal features between samples with positive and negative results for HHV1-5, AAV and HPV genomes detection.

	Presence of HSV1/2, VZV, EBV, CMV, AAV or HPV genomes		
	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.

VZV DNA, but no significant difference was found between semen parameters of positive and negative samples.

Sexual transmission is a route of EBV infection (Bezold et al., 2007; Kapranos et al., 2003), so the effect of EBV seminal infection on fertility was investigated in different studies. However, no relationship was found between sperm quality and detection of EBV DNA in semen. The genome of EBV was not detected in any of our samples in this study.

Naumenko et al. (2011) found that HCMV may disturb sperm maturation by direct toxic impact. Nevertheless, the other studies failed to show this association. A cross-sectional study by Kapranos et al. (2003) detected HCMV DNA in 7.1% of semen samples. However, no association was found between HCMV infection and sperm characteristics. In 2009, a study of 170 infertile men by Eggert-Kruse et al. (2009) showed that the presence of HCMV in semen did not significantly affect the quality of sperm. Besides, in a case-control study on semen samples in Iran, HCMV DNA was detected in 6% of infertile and 4% of fertile men respectively (Baghdadi, Tafvizi, & Hayati Roodbari, 2016). This is similar to the present study which showed no association between infertility and HCMV seminal infection.

For the first time a case–control study found AAV DNA in 30% of infertile men (30 case samples) but failed to find it in fertile men (8 control samples) concluding that the presence of AAV in semen can affect sperm motility (Rohde et al., 1999). Moreover, Erles et al. (2001) performed a case-control study on 73 infertile and 8 fertile men and found AAV DNA in 38% of semen samples from men with fertility problems. In contrast, a cross-sectional study on 146 semen samples showed the presence of AAV DNA in 19.9% of them but did not find any significant association with fertility (Schlehofer, Boeke, Reuland, & Eggert-Kruse, 2012). Similarly, in this study, 27.6% of semen samples were positive for AAV and no association was found between AAV seminal infection and semen quality.

HPV is one of the most important viral infections in genital tract of both men and women; its involvement in infertility had been investigated in different studies. For example, it was shown that sperm motility was decreased by incubation with HPV DNA E6-E7 region for 24 hours (Kim et al., 2012). Correspondingly, in a case–control study, Foresta et al. (2010) compared the detection rate of HPV DNA in semen samples of infertile and fertile men and found no relationship between HPV and semen parameters except for sperm motility which showed significant reduction in HPV positive cases.

In a large cohort of 430 male partners of couples seeking fertility evaluation, HPV-DNA was commonly present in semen (14.9%). There was no statistically significant association between the presence of HPV in semen and seminal parameters (Luttmer et al., 2016). Some of the other investigators reported that the HPV DNA detection in semen was not correlated with variations in sperm features, i.e. sperm counts and motility (Garolla et al., 2013; Rintala, Grénman, Pöllänen, Suominen, & Syrjänen, 2004). Similarly, in this study no correlation was found between seminal HPV DNA detection and changes in semen parameters. The prevalence of HPV DNA regardless of fertility disorders in 145 semen samples collected from private medical laboratories in Tehran was 19.3% (28 out of 145 cases). None of the attendants reported the history of genital warts. The rate of HPV DNA positivity in 90 semen samples in another cross-sectional study in Iran was 28.8% (26 out of 90 cases) (Nasseri et al., 2015). Moreover, the seroprevalence of HPV in 162 men aged 10–25 years in Tehran was assessed by Aghakhani et al. (2016) and the results showed that 22.2% of participants were seropositive for HPV. We initially designed this study to use a sensitive method for detection of some viruses in semen samples, but we did not determine the viral load of the samples. Despite the fact that viral quantitation might be critically important we will consider it for the future studies.

In conclusion, no association was found between semen quality and the detection of HHV1-5, AAV and

HPV DNA in semen samples. It should be noted that the prevalence of different viruses in semen, and their relevance to male infertility, differs significantly due to the genome extraction and amplification methods or due to a real variation between study populations and geographical regions.

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Disclosure statement

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