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The evaluation of Human papilloma virus and human herpes viruses (EBV, CMV, VZV HSV-1 and HSV-2) in semen samples

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Abstract

There are a number of risk factors, especially viral diseases, which can lead to infertility. Among the various viral infections, much attention has been given to the role of the Papillomaviridae and Herpesviridae. After collecting 82 semen samples (37 teratospermia, 2 asthenozoospermia, 2 oligoasthenospermia, 1 oligospermia, 6 asthenoteratospermia and 34 normal semen samples), and washing them, the DNA from both freshly ejaculated spermatozoon and washed spermatozoa was extracted. Subsequently, the prevalence of EBV, CMV, HSV-1, HSV-2, VZV and HPV was evaluated using Multiplex PCR and Nested PCR. In this study, 1 normal and 5 abnormal semen samples were infected with HSV-1 (1 normal, 4 teratospermia and 1 oligoasthenospermia). In addition, there were 2 VZV-positive samples (both were teratozoospermia). Nested PCR indicated that 1 asthenozoospermia, 1 asthenoteratospermia, 3 teratospermia and 4 normal samples were HPV positive (including 8 HPV-18 and 1 HPV-33). Among 9 HPV-positive subjects, 3 samples were negative after washing the infected samples. The prevalence of EBV, CMV, VZV, HSV-1 and HSV-2 remained unchanged prior to and after washing. Maybe sperm washing can be useful to eliminate HPV infection from semen samples, but further investigation is required because of the small number of samples.

KEYWORDS
CMV, EBV, HPV, HSV, VZV

1 | INTRODUCTION

Infertility is one of the serious problems worldwide, which can have effects on one-sixth couples (Brugo-Olmedo et al., 2001). Infertility means that couples cannot become pregnant after 12 months of trying to achieve a clinical pregnancy. Women who can get pregnant but cannot maintain a healthy pregnancy may be considered infertile as well (Nygren et al., 2011). The prevalence of infertility has been reported differently, ranging from 3.5% to 16.7% in more developed countries, and from 6.9% to 9.3% in the less developed ones (Boivin et al., 2007). The leading cause of infertility is not clear, but it is assumed due to different risk factors, including smoking, alcohol consumption, changes in sexual behaviour, and even infectious diseases, meaningful increase in the prevalence rate of infertility can be observed. Among different infectious risk factors, the role of various viruses has attracted much attention (Abedi et al., 2018; Arefzadeh et al., 2020; Boeri et al., 2019; Garolla et al., 2013; Ghasemnejad et al., 2018; Khajehzadeh et al., 2018; Mahmoudi et al., 2019; Mousavi et al., 2020; Nasiri et al., 2020; Pormohammad et al., 2019; Pourentezari et al., 2016; Schuppe et al., 2017).

Humanpapilloma viruses (HPV) are nonenveloped DNA viruses, belonging to Papillomaviridae. These viruses often infect epithelial cells, and they are one of the considerable risk factors which can cause infertility. These viruses have different types, and HPV16

and −18 are genotypes that are considered high-risk because of their higher oncogenic potential in (cervical, at first, but also others) epithelia. These high-risk viruses can encode E6 and E7 (Fan & Shen, 2018; Golrokh Mofrad et al., 2020; de Sanjosé et al., 2007). E6 has the potential to change the function of P53 tumour suppressor protein; therefore, it has a disruptive effect on the control of cell division (Crook et al., 1991). Furthermore, E7 separates Rb from E2F which initiates the over-expression of cellular protein (Cai et al., 2018; Crook et al., 1991; de Sanjosé et al., 2007; Lai et al., 1996).

Furthermore, Herpesviridae is assumed to be one of the major risk factors for infertility. This virus family comprises eight members, including herpes simplex virus type 1 and 2 (HSV-1, HSV-2), Varicella zoster virus (VZV), Epstein–Barr virus (EBV), human herpesvirus 6 and 7 (HHV-6, HHV-7), Cytomegalovirus (CMV) and Kaposi's Sarcoma herpesvirus (HHV8) (Ptaszyńska-Sarosiek et al., 2019). Although these viruses share many strategies, each has developed unique mechanisms in different diseases, for example, recurrence and latency of Herpesviridae are well-established. There is a strong relationship between Herpesviridae and various health disorders (Theil et al., 2002). For instance, researchers have acknowledged that EBV can cause different diseases, especially gastric cancer. Perhaps, Epstein–Barr Virus nuclear antigen (EBNA-1) has the ability to disrupt the cell cycle and reshape the cellular structure of spermatozoa (Faghihloo et al., 2014; Michou et al., 2012).

As clinical experiments have pointed to evidence that there is a link between viral infections and infertility (Garolla et al., 2013; Schuppe et al., 2017), we attempted to evaluate the prevalence of EBV, VZV, CMV, HSV-1, HSV-2 and HPV in semen samples.

2 | MATERIALS AND METHODS

2.1 | The patient's characteristics

The Ethics Committee of Shahid Beheshti University of Medical Sciences approved the study; IR.SBMU.RETECH.REC.1398.431 (Grant no 19225). Into a sterile specimen cup, 82 semen samples were collected from Taleghani hospital, Tehran, Iran, in 2019. Actually, semen samples were collected after 3–5 days of sexual abstinence, and semen parameters, especially sperm motility, sperm concentration ($\times 10^6$ sperm/ml) and sperm morphology were analysed according to the World Health Organization (WHO) guidelines Cao et al., (2011). The inclusion criterion in this study was BMI between 18 and 25 kg/m^2. Every patient experiencing radiation therapy, or chemotherapy was excluded from the study. Moreover, samples from patients suffering from varicocele, or experiencing previous testicular surgery, endocrine disorders or cancer were ignored. Every specimen was negative for bacterial infections, especially Mycoplasma genitalium, Chlamydia trachomatis and Neisseria gonorrhoeae. The consent for publication was obtained from all patients.

2.2 | Double washing of SPERM

We diluted a fraction of the sample (1:2) in human tubal fluid (HTF) medium (Geneocell; Iran) supplemented with 10% human serum albumin (HSA) (Nova Biologics; Australia). Then, the diluted sample was centrifuged and pelleted at 300 g for 10 min. After discarding the supernatant, the sperm pellet was resuspended in 1 ml of HTF medium + 10% HSA by gentle pipetting, and centrifuged at 300 g for another 10 min. After resuspending samples in 1 ml of HTF medium + 10% HSA, all samples were sent to virology laboratory to evaluate HPV, HSV-1, HSV-2, EBV, CMV, VZV prevalence, and investigate different genotypes of HPV (Björndahl, 2013).

2.3 | DNA Extraction

The phenol-chloroform protocol was used to extract DNA. First of all, samples were incubated in 300 μl of digesting buffer, containing 15 μl of proteinase K, at 37^{oC} overnight. Then, we removed protein with phenol (CinnaClon Co.), phenol-chloroform, and chloroform. Actually, 500 μl of phenol was added to an equal volume of sperm sample, and after centrifuging at 5,000 g for 5 min, 500 μl of phenol-chloroform was mixed with supernatant in a new tube. This process was followed by another centrifuging at 5,000 g for 5 min; then 500 μl of chloroform was added to supernatant in another new tube. After that, this mixture was centrifuged at 5,000 g for 5 min, and 1,000 μl of ethanol was mixed with supernatant and incubated overnight at −4 ^{oC} in a new tube. At the final stage, after centrifuging at 12,000 g for 30 min at 4 ^{oC} and discarding the supernatant, the DNA of sample was dissolved in 50 μl sterile water.

2.4 | PCR for β-globin gene

To confirm the quality of extracted DNA, we performed PCR for β-globin gene in final 25 μl volume, including 1 μl forward and 1 μl reverse primer (10 pmol), 12.5 μl master mix, 8.5 μl sterile water and 1 μl DNA. The PCR schedule was 95$^{°C}$ as first denaturation for 5 min, 30 cycles of 95$^{°C}$ for 30s, 55$^{°C}$ for 30s, 72$^{°C}$ for 30s and 72$^{°C}$ for 7 min. The PCR product was 100bp, and every positive sample was analysed to detect HSV1, HSV2, EBV, CMV, VZV and HPV. We double-checked every sample to confirm the quality of DNA extraction.

2.5 | Multiplex PCR

To evaluate the Herpesviridae infection, we set up a multiplex PCR. The PCR products were 269 bp, 715 bp and 934 bp for HSV1, HSV2 and VZV respectively. 50 μl was the final volume for multiplex PCR, including 25 μl master mix, 20 μl sterile distilled water, 2 μl DNA template, 1.5 μl forward and 1.5 μl reverses primers (table 1). The PCR reactions were performed using the Bio Intellectica PCR under

TABLE 1 Nucleotide sequences of primers used for Multiplex PCR and Nested PCR

PCR-HSV1-F	5′-GACTCTCCCACCGCCATCAG–3′
PCR- HSV1-R	5′-TGTCTTCGGGCGACTGGTCT–3
PCR- HSV2-F	5′-TATGCCTATCCCCGGTTGGA–3′
PCR - HSV2-R	5′-CGTGCCATCCGAATAAACGTG–3′
PCR-VZV-F	5′-TTGTGTCGGTCTCTCCAAGC–3′
PCR-VZV-R	5′-TACGTCTTCAACCTCACGCC–3′
PCR-CMV-F	5′-TGGCTTTTCTTGAACGTGCG–3′
PCR-CMV-R	5′-CCTTGACGCTGGTTTGGTTG–3′
EBNA F	5′-TGAATACCACCAAGAAGGTG–3′
EBNA R	5′-AGTTCCTTCGTCGGTAGTC–3′
MY–9	5′-CGTCC(A/C)A(A/G)(A/G)GGA(A/T) ACTGATC–3′
MY–11	5′-GC(A/C)CAGGG(A/T)CTATAA(C/T) AATGG–3′
GP–5	5′-TTTGTTACTGTGGTAGATACTAC–3′
GP–6	5′-AAAAATAAACTGTAAATCATATTC–3′
GAPDH F	5′-ATGTTCGTCATGGGTGTGAA–3′
GAPDH R	5′-GGTGCTAAGCAGTTGGTGGT–3′

the following conditions: 95°C for 5 min as an initial denaturation step, followed by 40 cycles at 95°C for 30 s, 58°C for 40 s, and 72°C for 1 min and 15 s, with a final extension at 72°C for 10 min. Then, all DNA products were run into 1.5% gel electrophoresis.

To evaluate the EBV and CMV infections, PCR analysis was performed to assess the presence of EBNA-1 and Glycoprotein gB genes respectively. In the final volume of 25 μl, we mixed 12.5 μl master mix, 7.5 μl of sterile distilled water, 1 μl of forward, 1 μl reverse primers and 3 μl of DNA. The PCR test was performed under the following PCR schedule: 5 min at 95°C, then 30 cycles of 95°C for 30s, for 30s at 55°C, for 30s at 72°C and for 10 min at 72°C. The PCR products for EBV and CMV were 400bp and 921 bp respectively. We selected the glycoprotein gB, glycoprotein gG and glycoprotein gB to detect CMV, HSV-1,2 and VZV respectively. DNA from the B-95–8 cell line was used as a positive control (for EBV) and sterile water was employed as a negative control. In fact, we cloned all these glycoprotein genes in Bcl-2 Escherichia coli, and used as positive controls. Every semen sample was double-checked.

2.6 | Nested PCR

For amplifying HPV L1, the combinations of 12.5 μl PCR master mix, 2.5 μl reverse primer called MY-11 and 2.5 μl forward primer named MY-9 (Table 1), 2.5 μl DNA, and 5 μl sterile water were used to make the first step of nested PCR. The first step of nested PCR schedule was 5 min initial denaturation at 94°C, 40 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min. After that PCR product (5 μl) was used in second round as a DNA template with 25 μl master mix, 2.5 μl forward primer (GP5), 2.5 μl reverse primer (GP6) and 15 μl sterile water. The second PCR schedule was 4 min initial denaturation at 94°C, 40 cycles of denaturation at 94°C for 1 min, annealing at 40°C for 2 min, extension at 72°C for 2 min and final extension at 72°C for 4 min. After that, the final PCR product (150 bp) was run into 1.5% agarose gel electrophoresis. HeLa cell line and sterile water were employed as positive and negative control respectively. All PCR reactions were double checked.

2.7 | Sequencing

After purifying all PCR products, forward direction was sequenced with ABI PRISM 310 genetic analyser (PE Applied BioSystems Inc) and ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (PE Applied BioSystems Inc).

2.8 | Analysis of phylogenetic

The results of HPV L1 sequences were aligned using CLUSTAL W tool in MEGA 6.0.6 with reference sequences from the GenBank database. After that neighbour-joining was employed to analyse phylogenetic tree. All the results were submitted in Gen Bank database and the accession numbers of them were MN689561 - MN689569.

2.9 | Statistical analysis

By the chi-square test, we analysed the results to assess the association between age and the prevalence of HPV, HSV-1, VZV. In terms of semen analysis, Shapiro–Wilks tests were used to investigate the normality of data. In addition, one-way ANOVA and Tukey's post hoc compared the semen parameters in different groups. All statistical analyses were performed using SPSS software, version 22.

3 | RESULTS

We collected 37 teratozoospermia, 2 asthenozoospermia, 2 oligoasthenozoospermia, 1 oligozoospermia, 6 asthenoteratozoospermia and 34 normal semen samples to evaluate HPV, HSV-1, HSV-2, EBV, CMV and VZV prevalence. After confirming the accuracy of DNA extraction, our results showed that 1 normal and 5 abnormal semen samples were infected with HSV-1 (1 normal, 4 teratozoospermia and 1 oligoasthenozoospermia) (Figure 1). In addition, there were 2 VZV-positive samples (both were teratozoospermia), (Figure 2) but we did not find any HSV-2, EBV and CMV positive sample. In this study, nested PCR indicated that there were 1 asthenozoospermia, 1 asthenoteratozoospermia, 3 teratozoospermia and 4 normal HPV-positive sperm samples (Figure 3). As HPV may interfere with sperm production, we attempted to assess different strains of HPV. Our results illustrated that there were 8 HPV-18 (3 normal, 3 teratozoospermia, 1 asthenozoospermia and 1 asthenoteratozoospermia

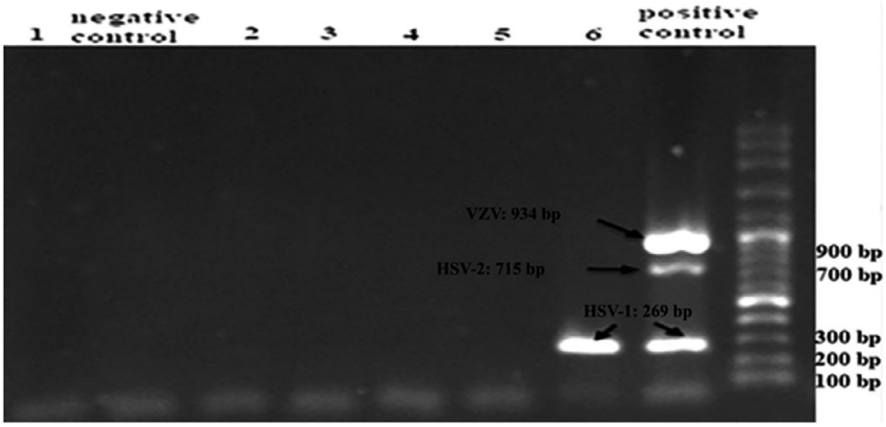

FIGURE 1 The final PCR products for HSV-1, HSV-2 and VZV were 269 bp, 715 bp and 934 bp respectively. Negative control has no DNA sample, and Positive controls of glycoprotein G (gG) were cloned in the plasmid

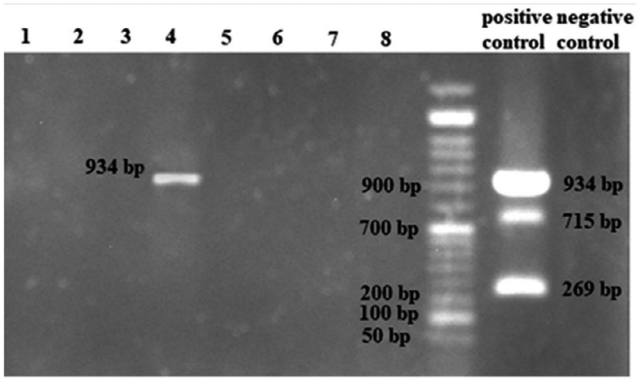

FIGURE 2 VZV final PCR production was 934 bp, and Glycoprotein gB was selected as a positive control

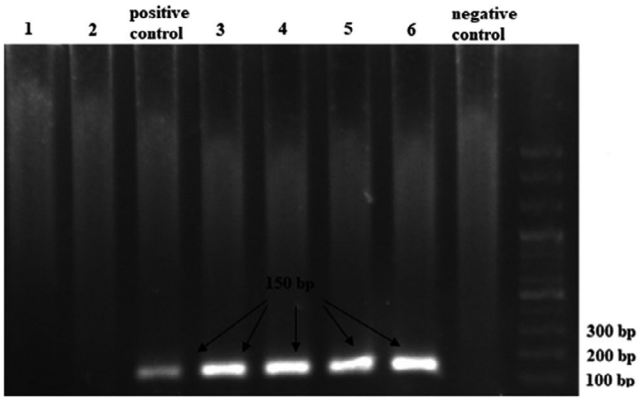

FIGURE 3 The final PCR product size for HPV nested PCR was 150bp. HeLa cell line DNA and sterile water were used as positive and negative controls respectively

HPV-18 positive samples) and 1 HPV-33 (1 normal sample). Figure 4 showed the phylogenetic tree of HPV. Among 9 HPV-positive subjects, 3 HPV-18 samples (3 normal sperm samples) were negative after washing. We did not find any changes in the prevalence of HSV-1, HSV-2, EBV, CMV and VZV before and after washing; moreover, we evaluated associations between HSV-1, VZV, HPV infections and the age of patients, and no meaningful relationship between them was observed. The percentage of sperm morphology in the HSV-1 and HPV-positive subjects showed meaningful decreases in comparison with the control (negative) group ($p < .05$). Also, all parameters of infected samples are summarised in table. 2.

4 | DISCUSSION

Infertility may be one of the major health problems in the world. Although many studies have attempted to evaluate the prevalence of different infectious risk factors, the prevalence of viral ones in sperm specimen is not clear. It is supposed that viral infections can accelerate the progression of different kinds of diseases, for example, previous studies have reported that HPV E6 is able to downregulate the expression of p53 (Abbasi et al., 2020; Foresta et al., 2013; Ghasemnejad et al., 2019; Hemmat & Baghi, 2018; Hu et al., 2011). Furthermore, there are numerous relationships between Herpesviridae, such as EBV, and different kinds of health problems, especially gastric and breast cancer (Aris et al., 2020; Golrokh et al., 2020). Maybe EBNA-1 is able to disrupt the cell cycle, and it can be responsible for changing the cellular structure, such as sperm cells (Faghihloo et al., 2014; Garolla et al., 2013; Kaspersen et al., 2012).

Size and volume of sample are one of the important parts of an epidemiological study. The limitations of this study were small number of sample and low semen volume. In this study, we detected 1 normal, and 5 abnormal semen samples infected with HSV-1. In addition, there were 2 VZV-positive samples (both were teratozoospermia), and none of the samples showed the CMV, HSV-2 and EBV infections. There were no changes in the prevalence of EBV, CMV, VZV, HSV-1 and HSV-2 before and after washing. Our data suggest that HSV-1 infection can cause a significant decrease in normal sperm morphology (Table. 2).

The presence of these viruses in male genital tract may reduce semen quality and male fertility through germ cell infection, local inflammation, and the disruption of the blood-testis barrier. (Fijak & Meinhardt, 2006; Sabeti et al., 2016; Salam & Horby, 1922) This barrier is composed of tight junctions between the basal surface of adjacent Sertoli cells, isolating sperm antigens from the immune system. When the barrier is disrupted because of local inflammation, an

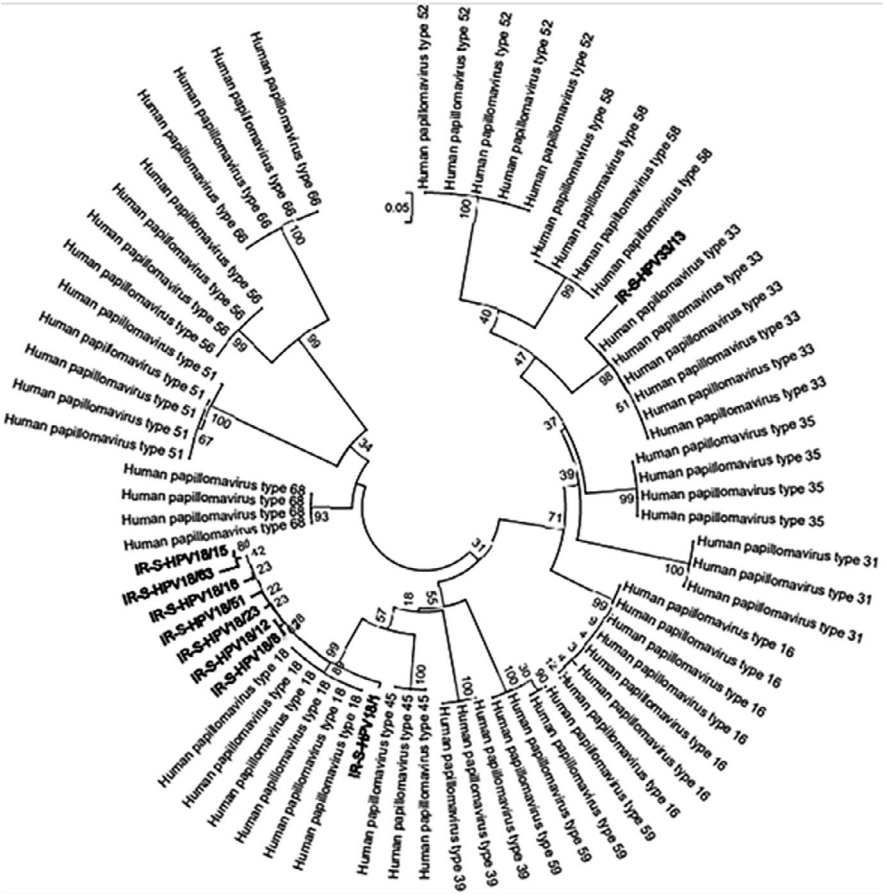

FIGURE 4 HPV L1 gene phylogenetic relationships of the sequences isolated from semen samples. The MEGA 6.0.6 was used for construction of neighbour-joining tree by Kimura 2-parameter model with bootstrap of 1,000 replicates. We highlighted the sequences in this study

TABLE 2 Semen parameters in virus-positive groups (HPV, HSV-1, VZV) compared with control group

Semen parameters	Control ($n = 65$) (control negative group)	HPV ($n = 9$)	HSV−1 ($n = 6$)	VZV ($n = 2$)
Sperm concentration (10^6 /ml)	83.3 ± 9	68.9 ± 17.7 $p = .873$	54.5 ± 15.5 $p = .578$	17.5 ± 2.5 $p = .294$
Total sperm motility (%)	58.8 ± 1.7	48.3 ± 5.3 $p = .139$	51.8 ± 8.4 $p = .604$	27.5 ± 2.5* $p = .008$
Progressive sperm motility (%)	47 ± 1.5	37.3 ± 3.9 $p = .107$	44.5 ± 8 $p = .954$	13 ± 2* $p = .001$
Normal sperm morphology (%)	6.3 ± 0.7	2.3 ± 0.6 * $p = .023$	1.9 ± 0.6* $p = .037$	0.5 ± 0.5 $p = .128$

Note: Sperm concentration: number of sperm/mL in a semen sample. Total sperm motility (%): the total percentage of spermatozoon that are actively moving or shaking in ejaculate. Progressive sperm motility (%): percentage of spermatozoon moving actively, either linearly or in a large circle. Normal sperm morphology (%): percentage of spermatozoon with normal shape of head, mid-piece, and tail. Note. Data are presented as mean ± *SEM* and analysed by the one-way analysis of variance (ANOVA) and Tukey's post hoc.

*$p < .05$, compared with control group.

immune response is elicited, causing spermatogenic failure. In addition, local inflammation during sperm migration can cause immune response and generation of reactive oxygen species (ROS) which can lead to sperm membrane damage, DNA fragmentation, and abnormal sperm morphology and motility (Fijak & Meinhardt, 2006; Sabeti et al., 2016; Salam & Horby, 1922).

Although some studies have focused on HPV, EBV, CMV, VZV, HSV-1 and HSV-2, the prevalence of these viral infections in sperm sample is not clear. In 2001, 25% of semen donors had HSV-1 infections, 16.7% were infected with both HSV-1 and HSV-2 simultaneously, and there was no HSV-2 infection exclusively (Neofytou et al., 2009). In Brazil, between November 2014 and July 2015, 7.5% of semen samples had HSV-1, and 3.2% had HSV-2 exclusively. Kurscheidt indicated that there is a tight relationship between HSV-1 infection and low sperm count. HSV-2 infection may lead to haematospermia and low semen volume, as well

(Kurscheidt et al., 2018). In another study, viral cultures illustrated that 9% of samples were infected with HSV-2 DNA. In addition, CMV DNA was present in 6.3% of the semen samples (Aynaud et al., 2002). In a study by Kapranos et al., using nested PCR, 64 (56.6%) of the 113 semen samples were found to be infected with different DNA viruses (49.5%, 16.8%, and 7.1% of sperm samples were HSV, EBV, and CMV positive respectively). Furthermore, lower sperm count, and poor motility were associated with HSV infection (Kapranos et al., 2003). Among 83 semen samples of infertile men, 8 and 4 specimens were CMV and HSV-2 positive respectively. However, there was no sample coinfected with both of these viruses (Wu et al., 2007). HSV DNA was detected in 46% of samples (HSV-1 in 21 cases (26%) and HSV-2 in 16 cases (20%)). In addition, the rate of HSV infection was three times higher in the samples, with sperm concentration lower than 20 million/ml. In another study, sperm count and motility for HSV positive samples were reported about 23.5% and 36%, respectively, but these items for HSV negative specimens were 53.2% and 47% respectively (P(count) = 0.0005 and P(motility) = 0.01) (Kotronias & Kapranos, 1998). Immunofluorescence (IF) reaction showed 13 samples (65%) had HSV antigen; furthermore, it was reported that HSV may have a detrimental effect on sperm function and pregnancy (Bocharova et al., 2007). In another study, 27.2% of samples were HSV-1 positive, and in 0.4%, 0.1%; 6.3%, 2.7%, 13.5% and 4.2% of samples HSV-2, EBV, CMV, HHV-6A/B and HHV-7 DNA were detected respectively (Kaspersen et al., 2012).

In this study, nested PCR indicated that there were 1 asthenozoospermia, 1 asthenoteratozoospermia, 3 teratozoospermia and 4 normal HPV-positive sperm sample. Among 9 HPV-positive subjects, 3 HPV-18 (3 normal semen samples) were negative after washing our infected specimens. Sperm washing is a procedure which can reduce infection load, and increase the chance of infertility treatment by removing mucus and nonmotile sperm. Some studies have reported that the sperm-washing techniques can eliminate different viral infections, for example, Garolla suggested all HPV, HSV, HCMV, HBV, HCV, HIV and AAV infections have a negative effect on male reproductive system, and sperm washing can avoid viral transmission (Garolla et al., 2013). In 2012, it was reported that washing technique can reduce HPV DNA, completely (Garolla et al., 2012). But some reported that the semen washing procedures cannot remove HPV from infertile samples (Foresta et al., 2011). In this study, sperm morphology percentage of the HPV-positive subjects showed significant decreases in comparison with the control group (Table. 2). Some studies have confirmed our results, for example, in 2019, 8 (11.43%) out of 11 cases of infertile men were HPV positive; however, none of the fertile samples were HPV positive (Moghimi et al., 2019). 23.9% (n = 54,226) of samples had HPV in sperm samples, and miscarriage rate was higher in HPV-positive samples (Garolla et al., 2016). In 2013, Yang Yang showed 12.48% of subjects were HPV positive (among 523 fertile males, only 6.70% had HPV infection). Also, there was a decrease in progressive motility (PR) and the normal morphology percentage in HPV-positive samples (Yang et al., 2013). A study conducted by Bossi in Brazil between March and August 2016, HPV DNA was observed in 28% of specimens, and sperm motility was higher in HPV-positive semen samples (Bossi et al., 2019). Fluorescence in situ hybridisation (FISH) analysis indicated that 25% of sperm samples had the DNA of HPV at the head site; however, the integration of HPV in the nucleus was not clear (Foresta et al., 2010). In another study, 24 out of 45 infected specimens, and 3 out of 40 healthy samples illustrated the presence of HPV DNA. Swim-up also showed the proportional reduction in the HPV DNA (in 2 cases) (Olatunbosun et al., 2001). In 2004, 15.4% of samples had high-risk HPV DNA, but volume, sperm concentration, motility and vitality of spermatozoa were not affected. It was reported, the PH of HPV-positive samples was lower than negative ones (Rintala et al., 2004). In 2010, 40.9% of infertile patients, 10.2% of fertile controls and 2.2% of infertile patients were infected with HPV. In addition, there was a reduction in sperm motility in HPV-positive samples (Foresta et al., 2010).

5 | CONCLUSION

In this study, the prevalence of HPV, HSV-1 and VZV in abnormal semen samples was higher than normal ones. 8 and 1 samples were infected with HPV-18 and HPV-33, respectively, and 3 samples were negative after washing the HPV-18 infected samples. Maybe semen washing can be useful to eliminate HPV infection from samples, but further investigations are required because of small number of samples.

ACKNOWLEDGEMENTS

The Ethics Committee of Shahid Beheshti University of Medical Sciences approved the study; IR.SBMU.RETECH.REC.1398.431 (Grant no 19225).

CONFLICT OF INTERESTS

The authors declare no conflicts of interests.

AUTHORS' CONTRIBUTIONS

Shaian Tavakolian, Hossein Goudarzi, Hamid Nazarian, Sarah Niakan, and Pourya Raee designed the study and performed the molecular experiments. Ebrahim Faghihloo performed the statistical Analyses.

ETHICAL APPROVAL

The Ethics Committee of Shahid Beheshti University of Medical Sciences approved the study; IR.SBMU.RETECH.REC.1398.431 (Grant no 19,225).

CONSENT FOR PUBLICATION

The written consent for publications was recieved from all patients.

DATA AVAILABILITY STATEMENT

The data that support the finding of this study are available from the corresponding author upon reasonable request.

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