Original article

Prevalence and types of anal hpv in infertile patients with semen infection

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Abstract: Introduction. HPV infection is a very common sexually transmitted disease. Studies regarding anal infection in men, typically focus on HIV-immunosuppressed subjects or men who have sex with men. *Objective*. To evaluate the prevalence of anal HPV in infertile patients with infected semen and to detect the genotype concordance and most prevalent types at both sites. *Methods*. In 118 infertile patients with positive FISH analysis for HPV on semen, sperm parameters were compared with those of 172 infertile noninfected men serving as control group for semen analysis. In the former group, detection and genotyping of HPV infection was performed by INNO-LiPA assay in semen and anal brushes. *Results*. Sperm parameters from infected males showed significantly reduced motility and increased anti-sperm antibodies compared with controls (P<0.001 and P<0.01). HPV DNA detection and typing in semen showed HPV-16 as the most prevalent genotype (24.6%). Anal brushing tested positive 47 subjects (39.8%) showing high concordance with seminal HPV types. In this group, 7 patients (14.9%) had anal warts and 23.4% had a coinfection by more HPV types. Most prevalent HPV type observed in patients with anal infection was HPV-6 (24.4%). *Conclusions*. This study demonstrates for the first time that HPV semen infection may represent a risk factor for anal HPV even in heterosexual asymptomatic men. Because the strong associacion between anal cancer, anal warts and HPV infection, we suggest testing the anal site of patients with semen infection and to counsel them in order to prevent warts and cancer development.

Key words: Anal HPV; Heterosexual males; HPV Semen infection; Male infertility; Sexual transmitted disease.

INTRODUCTION

Human Papillomavirus (HPV) is the most common sexual transmitted disease (STD) worldwide. HPV are small non-enveloped DNA viruses able to infect mucosal and cutaneous membranes of the anogenital region, upper aerodigestive tract, and other head and neck mucosal regions[1]. This infection is mainly asymptomatic and transient[2], however it may persist and give rise to various lesions such as warts[3], low grade squamous intraepithelial lesion (LSIL), high grade squamous intraepithelial lesion (HSIL), and invasive cancers[4,5] depending on low or high risk (LR or HR) type of HPV infection. This infection has been largely investigated in women and it is estimated that about 10% of women worldwide with normal cytological screening, tested positive for cervical HPV[6]. Moreover, HR HPV type's infection represents a well known cause of invasive cervical cancer, which is the second most common cancer among women[7]. Despite HPV prevalence results higher in females, recently HPV has been taken into account for men's health and at present, there is a growing interest in further understanding the relationship between HPV infection and disease in men, including the development of genital warts, penile cancer, anorectal cancer and oropharingeal cancer[8]. Recently, new insights on human reproduction suggested a major role for HPV even in male infertility[9-12]. The reported prevalence of genital HPV DNA in men has a wide range, from 1.3% to 72.9% with most studies showing ≥20%[13,14]. Recently, it has been reported that HPV can be found not only along the whole male genital tract but even in semen[15]. Interestingly, the presence of HPV DNA at this site has been demonstrated to be associated with an impairment of sperm motility and presence of anti-sperm antibodies[16,17]. In the last years, it has been observed a steady increase of this sexual transmitted disease (STD) and it is currently the most common STD seen by colorectal surgeons for anal lesions, with a million new cases seen every year[18]. It is known that about one-third of sexually active young women have a detectable HPV infection on anal smear, with a higher prevalence among those who reported anal intercourse[19]. In men, who have sex with men (MSM) the reported prevalence of anal HPV is about 57 percent, with HPV-16 being the most common type[20]. In heterosexual men, the prevalence of anal HPV is about 12 percent[21,22] and known risk factors are a lifetime number of ≥ 10 female sex partners, a primary sexual relationship <1 year in duration, and a prior hepatitis B diagnosis[21]. While for women the presence of cervical HPV remains a major risk factor for anal HPV, even in the absence of anal intercourse[19,23,24], in heterosexual men the relation between HPV semen infection and anal HPV is still not fully understood. To evaluate this aspect, in the present study we tested for anal HPV, 118 consecutive subjects who had semen infection during the dignostic workup for infertility.

METHODS AND MAIN OUTCOME MEASURERS

Patients

Written informed consent was obtained from all patients, and the study protocol was approved by the local ethics committee, Protocol Number 2331P. Inclusion criteria: heterosexual infertile men that were tested positive for semen HPV at Fluorescent in situ hybridization (FISH) on the ejaculated semen for the detection of HPV-DNA sequences in the spermatozoa and exfoliated cells during the dignostic workup for infertility. Exclusion criteria: HCV, HBV, HIV infection, previous knowledge of HPV infection or previous HPV vaccination. We included in the study 118 consecutive infected patients. A medical history including smoking, sexual behavior and previous circumcision (in males) was obtained from each patient. At enrolment, all subjects were tested for semen analysis, detection of HPV DNA and typing on semen and anal brushing and were evaluated to detect the presence of anal warts. Sperm parameters were compared with an age matched group of 172 noninfected infertile men.

FISH for HPV on semen

As previously reported[25], glass slides containing at least $2x10^6$ adhered sperm were fixed in a methanol-acetic acid solution for at least 1 hour at −20°C. To permeabilize, samples were digested with pepsin diluted 1:25,000 in prewarmed 0.01 mol/L-1 HCl for 10 minutes at 37°C. Permeabilization of the specimens was stopped with 3- to 5-minute washes in PBS 1x;

then samples were dehydrated in 70%, 80%, and absolute ethanol for 2 minutes and finally air-dried. Samples were then overlaid with 20 mL of hybridization solution (Pan Path) containing biotin-labeled HPV DNA probe (a mix of total genomes containing the conserved HPV region). Each slide was covered with a glass coverslip, and the edges were sealed with nail polish to prevent loss of the mixture during denaturation and hybridization. After a simultaneous denaturation of cellular target DNA and HPV DNA probe on a heating block for 5 minutes at 95°C, hybridization was performed by incubating the samples at 37°C overnight in a humidified chamber. Thereafter the coverslips were carefully removed, and the slides were washed in PBS 1× for 10 minutes. After 15 minutes' incubation at 37°C with the differentiation reagent (Pan Path), the slides were washed three times in PBS 1x. The biotin-labeled HPV probe was detected by incubation with 1:200 streptavidin Texas Red (Vector Laboratories) for 40 minutes at room temperature. After detection the slides were washed twice in PBS 1x/0.01% Triton and thentwice in PBS 1x and mounted with a solution containing DAPI and anti-fade (BioBlue; BioView). Samples were analyzed using a fluorescence microscope (Nikon ViCo video confocal microscope) equipped with a triple band-pass filter set (FITC, TRITC, DAPI). For each slide at least 200 spermatozoa and 200 exfoliated cells were analyzed. Evaluation of nuclear hybridization signals was performed by three investigators. When nuclei were completely and homogeneously stained and multiple small spots or single large signals were present, the sperm cells were classified as positive. The method was tested on control slides containing CaSki cells, a human cervical carcinoma cell line with stably integrated and transcriptionally active HPV genomes that served as a control for the specific probe. Cells smeared on salinated glass slides were fixed with 4% paraformaldehyde in PBS for 10 minutes. After fixation, cells were subjected to 3- to 5-minute washes in PBS 1x and then dehydrated with 5-minute ethanol washes (30%, 60%, and 95%). Cell smears were then air-dried and stored at 4°C until use.

Semen Analysis

Semen samples were obtained by masturbation after 3 days of sexual abstinence. After liquefaction at room temperature, semen volume, pH, sperm concentration, viability, motility, and normal morphology were determined according to World Health Organization guidelines for semen analysis[26]. In each sample we performed also the spermMar test to sperm antibodies detection. Sperm antibodies were detected using the spermMar Test kit for IgG and IgA (FertiPro N. V., Sint-Martens-Latem, Belgium). Semen samples were treated according to the kit protocol. The test was considered positive when spermatozoa were partially or totally covered by latex particles. The reactivity of the test was confirmed by the next formation of growing agglutinates of latex particles themselves.

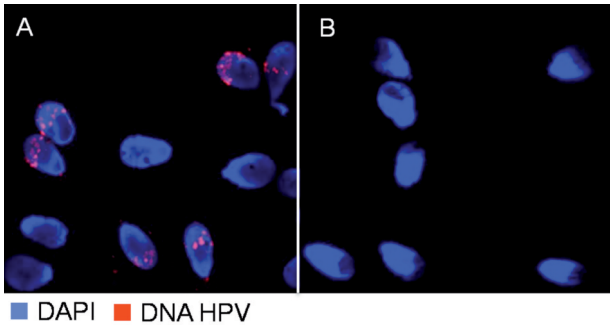

Figure 1. – Examples of FISH analysis for HPV performed in semen samples of infertile patients. A) Infected and B) non-infected semen sample. Red staining indicates the presence of HPV DNA.

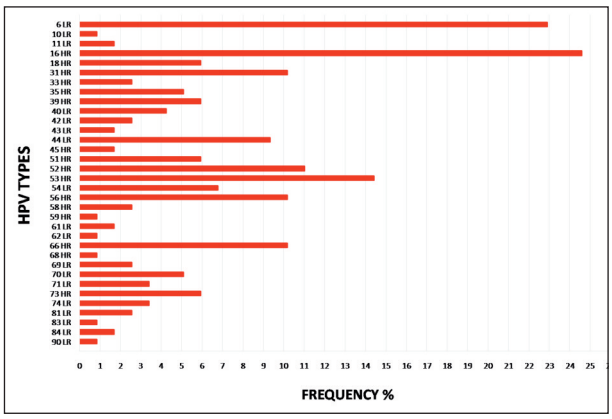

Figure 2. – Prevalence of high-risk (HR) and low-risk (LR) HPV types in semen samples of infertile men.

On the other hand freely moving spermatozoa uncovered by latex particles were considered as negative. A semen aliqot of 0.5 mL was used to perform HpV detection and typing.

Detection of HPV DNA and typing

A sample of exfoliated cells was obtained by a wide brushing of the anal region, using a standard-sized, COPAN ESwab™. Swabs were placed in phosphate-buffered saline, squeezed and rotated against the side of collection tube to release as much liquid as possible. Detection and typing of HPV on semen was performed using a 0.5 mL aliquot of whole semen. HPV DNA detection and typing was performed by using the INNO-LiPA HPV Genotyping Extra assay (Innogenetics, Ghent, Belgium) according to the manufacturers' protocol. For the INNO-LiPA test, total DNA was isolated from 200 µL of the other STM sample aliquot by using Qiagen DNA purification reagents on a Qiagen 3604 BioRobot (Qiagen) and eluted in 100 µl of water. Each DNA extraction run contained blank negative controls and positive controls to monitor the DNA isolation procedure. Five microliters of the DNA solution was used for PCR amplification using the INNO-LiPA HPV Genotyping Extra assay reagents. Biotinylated PCR products were genotyped by hybridization to HPV type-specific oligonucleotide probes bound to nitrocellulose membrane and detected by colorimetric reaction using an Auto-LiPA48 instrument in accordance with the manufacturer's recommendations. After colour development, strips were scanned and evaluated by the Line Reader and Analysis Software (Innogenetics) to determine HPV genotype/s. All results were confirmed by visual inspection. A sample was considered positive if at least one of the defined type-specific banding patterns or one of the HPV control lines were positive. Patients tested positive for anal HPV were invited to undego to colposcopy of the anal region.

Statistical analysis

The results are expressed as mean + SD and categorical variables are expressed as a percentage.

Comparisons between groups were performed with unpaired Student's t-tests after acceptance of normality with the Kolmogorov-Smirnov test and by the chi-square test for categorical data.

Probability (p) values of <.05 were considered statistically significant.

RESULTS

In this study, 118 infertile males (mean age 33.3+5.5) tested positive at FISH for HPV on semen during the dignostic workup for infertility, were further evaluated for anal infec-

TABLE 1. – Sperm parameters of infertile patients with HPV infected semen are compared with a group of noninfected infertile men. *P < 0.01 vs non-infected patients; **P < 0.001 vs non-infected patients.

Patients	Semen Volume (mL)	Sperm Concentration (mill/mL)	Sperm Count (mill/tot)	Motility (a+b) (%)	Normal Morphology (%)	Sperm Viability (%)	Sperm Antibodies n. (%)
HPV-infected Infertile patients n=118	2.4+1.4	55.7+49.1	143.8+131.9	21.7+15.6**	13.7+12.6	66.7+31.2	38 (32.2) *
Noninfected Infertile patients n=172	2.7+1.5	52.2+50.3	131.9+128.4	34.3+14.9	14.8+13.7	66.1+27.3	18 (10.5)

tion. All patients referred no symptom both at penile and anal level. Figure 1 shows examples of FISH analysis for HPV performed in semen samples of infected (A) and noninfected (B) infertile patients. In table 1 are reported sperm parameters observed in infected patients and in a group of age matched noninfected infertile subjects (mean age 34.6+4.7). Seminal volume, pH (data not shown), sperm concentration, sperm count, normal morphology, and viability were not different in HPV-infected and in noninfected infertile patients. A significant reduction of mean progressive sperm motility and a higher prevalence of antisperm antibodies was found in semen samples of infected patients compared with noninfected ones (respectively P<0.001 and P<0.01).

Patients with positive FISH for HPV at sperm level, underwent to DNA viral detection and typing in semen. This analysis allowed us to identify the following HR: HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and the following LR HPV genotypes: HPV-6, 10, 11, 40, 42, 43, 44, 54, 61, 62, 69, 70, 71, 74, 81, 83, 84 and 90. Coinfection by more HPV types (from two up to five types) was present in 54 patients (45.7%). The prevalence of each HPV type in semen is shown in figure 2. HPV-16 was the most observed infection (24.6%), followed by HPV-6 (22.6%). Other types, both those present in the quadrivalent vaccine and those types against which it offers a cross-protection (27), showed the following prevalence: HPV-11 1.7%, HPV-18 5.9%, HPV-31 10.2%, HPV-33 2,5%, HPV-45 1.7%, HPV 52 11.0% and HPV 58 2.5%). In these patients, the anal brushing for the detection of HPV DNA and typing tested positive 47 subjects (39.8%). Among these, 3 patients (6.4%) had anal warts at first examination and 4 more subjects (8.5%) showed anal warts at colposcopy. No dysplastic lesions were found. Most prevalent HPV types observed at anal site were: HPV-6, 16, 53, 56, 66, 44, 52 and 31 (respectively, 24.4, 18.6, 20.3, 17.7, 16.5, 10.1, 9.3 and 8.4% frequency). Coinfection by more HPV types was present in 23.4% of these subjects. Finally, we found a high concordance between HPV types observed in semen and those detected at anal brushing.

DISCUSSION

Clinically, HPV infection of the anal region can present as benign warts, dysplastic lesions or as a combination of both. Symptoms are frequently associated with the perception of a raised anal lesion, anal pruritis, bleeding and pain. In case of malignant forms, the disease shows growing lesions that tend to invade locally with possible formation of abscesses and fistulas and progression into carcinoma. Recently, several papers have established a causal relationship between HPV anal infection and the development of cancer at this site, even showing that 88% of these cancers are attributable to HPV[28]. However, in most cases HPV anal infection is represented by a silent infection and affected subjects are asymptomatic.

It is well known that HPV can be easily transmitted between sexual partners, mainly due to multiple transmission events that can occur between a couple[29]. It has been demonstrated that eighty-six percent of asymptomatic men whose sexual partners presented HPV infection, had positive HPV DNA detection by PCR at any genital site[30]. Although the role of males in the transmission of this virus to women seems clear, the prevalence of infection in the anal region of infertile men and associated risk factors have not been fully investigated. The presence of anal HPV infection has been usually related to the immunosuppression secondary to HIV infection and associated to MSM with a history of anal-receptive intercourse[31]. Up to now, no studies have considered the presence of HPV in semen as a risk factor for anal infection.

In the present study, infertile men with HPV semen infection were evaluated to test the prevalence of HPV at anal site. In particular, sampling the anal region enabled HPV detection in almost 40% of infertiles with infected semen, and high concordance of HPV types was observed at both sites. Recent studies reported that HPV infection can be found in about 12% of heterosexual men and in 50% of HIV-negative MSM having any type of anal HPV infection[28]. Considering these data, our results demonstrated a very high prevalence of anal HPV detection among infertile men with semen infection. As previously reported these patients had impaired sperm parameters[16,17], showing significantly reduced sperm motility and icreased prevalence of anti-sperm antibodyes. In semen, the most prevalent types were HPV-16 and HPV-6, with a prevalence of 24 and 22% respectively. At anal site, HPV-6 was the most prevalent (24.4%) followed by HPV-16 (18.6%). Anal coinfection by more HPV types was less frequent that in semen (23.4 versus 45.7%). Interestingly, three high risk (HPV 53, HPV 56, HPV 66) and one low risk type (HPV 44) against which the quadrivalent vaccine does not offer a cross-protection, were highly represented at anal brushing. Among patients with anal infection, about 15% showed warts at inspection or colposcopy.

CONCLUSIONS

Because the strong associacion and type concordance between seminal and anal HPV infection observed in our infertile patients, semen infection should be considered as a risk factor for anal HPV even in heterosexual asymptomatic men. Our data suggest that HPV could be spread from semen to anal region possibly during the personal care of genital area after sexual intercouse. Despite the lack of effective therapies for HPV infection, good reasons to screen anal HPV in in these patients are: i) high relation between HPV anal persistence and cancer at this site; ii) high incidence of anal warts that frequently lead to psychosexual consequences in affected patients[32]; iii) high prevalence of anal HPV types (both

HR and LR), against which quarivalent vaccine does not offer protection; iv) anal infection is considered an anatomic site that could act as viral reservoirs able to sustain the persistence of HPV infection[33]; v) possible transmission of the infection to the the female partner.

Although more and larger studies are needed to confirm our findings and to assess possible side effects of HPV anal persistence in these subjects, we suggest testing the anal site of patients with semen infection and to counsel them in order to prevent warts and cancer development.

Conflict of interest.

None to declare.

Autorship

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Analysis of data: Menegazzo M, Ghezzi M
Interpretation of data: Garolla A, Pizzol D, Foresta C.
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