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ARTICLE

Human papillomavirus in spermatozoa is efficiently removed by washing: a suitable approach for assisted reproduction

BIOGRAPHY

Claudio Fenizia obtained his PhD in Molecular Medicine of Immune and Inflammatory Reactions in 2009 before moving to the National Institutes of Health (Bethesda, MD, USA) to pursue his research on retroviruses and infectious disease. He recently moved to the University of Milan (Italy), as an Assistant Professor in immunology and pathology.

Claudio Fenizia[1,*,†], Cecilia Vittori[2,†], Monica Oneta[3], Bina Parrilla[3], Antonio Granata[4], Salomè Ibba[2], Mara Biasin[2], Mario Clerici[1,5], Daria Trabattoni[2], Valeria Savasi[3]

KEY MESSAGE

The sperm-wash procedure that was employed successfully removed human papillomavirus (HPV) from the motile-sperm fraction, which is used for assisted reproduction techniques. In light of this recent success, HPV screening should be routinely performed on sperm samples and, upon HPV positivity, sperm-washing should be performed before assisted reproduction techniques.

ABSTRACT

Research question: Is it possible, by sperm-washing spermatozoa from clinically HPV-positive men, to obtain spermatozoa free of human papillomavirus (HPV) to be employed in assisted reproduction?

Design: This was an observational study performed on HPV-positive men. Freshly ejaculated semen was collected and readily processed by gradient separation followed by swim-up from the washed pellet. The resulting fractions were seminal plasma, cell pellet, round cells, non-motile spermatozoa and motile spermatozoa. All fractions were then tested for the presence of HPV DNA.

Results: Of the 15 clinically HPV-positive subjects, 67% were positive in at least one of the seminal fractions. If any postivity was detected, the plasma was always HPV positive. No consistent pattern was observed throughout different samples in the cell pellet, round cell and non-motile spermatozoa fractions. However, after the sperm-wash procedure, the fraction of motile spermatozoa was never found to be HPV-positive.

Conclusions: The sperm-washing technique, which was previously successfully used to remove human immunodeficiency virus, can efficiently remove HPV from spermatozoa. However, the present study was conducted on a small population so a larger follow-up study is recommended. HPV screening should be performed in sperm samples and, upon HPV positivity, sperm-washing should be considered before assisted reproduction techniques are used.

[1] Department of Pathophysiology and Transplantation, University of Milan, Milan, Italy
[2] Department of Biomedical and Clinical Sciences, University of Milan, Milan, Italy
[3] Department of Obstetrics and Gynaecology, Sacco Clinical Sciences Institute, University of Milan Medical School, Milan, Italy
[4] Unit of Urology, Sacco Clinical Sciences Institute, Milan, Italy
[5] IRCCS Fondazione Don Carlo Gnocchi, Milan, Italy
[†]These authors should be regarded as joint first authors.

KEYWORDS

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*Corresponding author. E-mail address: claudio.fenizia@unimi.it (C.Fenizia). https://doi.org/10.1016/j.rbmo.2020.01.030
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INTRODUCTION

Human papillomaviruses (HPV) are a heterogeneic family of non-enveloped DNA viruses that commonly infect epithelia. Around 75% of the human population worldwide is estimated to be HPV positive (*Capra et al., 2015*; *Giuliano et al., 2011*; *Koutsky, 1997*; *Lowy et al., 1994*).

In addition to testicular cancer (*Garolla et al., 2012b*), HPV infection has been related to decreased fertility in male individuals (*Boeri et al., 2019*; *Damke et al., 2017*; *Donà et al., 2018*; *Foresta et al., 2010b*; *Garolla et al., 2012a*, *2013*, *2018*; *Gizzo et al., 2014*; *Luttmer et al., 2016*; *Moghimi et al., 2019*; *Tangal et al., 2019*; *Yang et al., 2013*), and to a decreased success rate during IVF (Depuydt et al., n.d.; *Garolla et al., 2016*; *Henneberg et al., 2006*; *Noventa et al., 2014*; *Pereira et al., 2015*). In fact, among couples undergoing assisted reproduction procedures, an increased risk of pregnancy loss is seen in presence of HPV infection and in particular when the male partner is infected (*Perino et al., 2011*). Confirming these findings, one of the predictive factors for abortion was indeed shown to be HPV positivity (*Perino et al., 2011*).

Although a steadily increasing literature is focused on the impact of HPV on spermatozoa (*Depuydt et al., 2019*; *Didelot-Rousseau et al., 2007*; *Giuliano et al., 2011*), it is still not clear whether HPV is prevalently localized in the seminal plasma or in the cellular fractions (*Capra et al., 2015*; *Cortés-Gutiérrez et al., 2017*; *Golob et al., 2014*). In fact, multiple authors report HPV positivity of the overall spermatozoa (*Foresta et al., 2010b*; *Garolla et al., 2016*; *Gizzo et al., 2014*; *Luttmer et al., 2016*; *Yang et al., 2013*), but only a few of them confirm HPV to be present inside spermatic cells *in vivo* (*Foresta et al., 2010b*, *2011a*, *2011b*). In fact, using fluorescence in-situ hybridization, Foresta and colleagues observed the presence of HPV DNA in the equatorial region of the sperm head in 25% of spermatozoa (*Foresta et al., 2010a*, *2010b*). Interestingly, HPV was never found in the spermatozoa of fertile subjects (*Foresta et al., 2010b*). Such observations are supported by Pérez-Andino and co-workers, who confirm the presence of HPV in the spermatozoon's head (*Pérez-Andino et al., 2009*). However, such observations were performed upon incubation with the virus, and are therefore meant as an in-vitro model. Recently, Capra and colleagues developed a new approach to evaluate the localization of HPV in the different cellular components of the semen (*Capra et al., 2019*). They observed the presence of HPV DNA, belonging to one or multiple HPV genotypes, in different seminal fractions.

Uncertainities about the actual localization of HPV in semen notwithstanding, the observation that HPV infection is associated with decreased fertility is universally accepted. This raises concerns for the role played by the virus within the context of assisted reproduction, especially considering that the prevalence of HPV-positive semen among sperm donors (*Foresta et al., 2010b*), and among those who bank their spermatozoa because of medical procedures (*Kaspersen et al., 2011*), is high. For this reason and because HPV-positive spermatozoa increase the risk of infection in women, the study set out to investigate the prevalent localization of HPV in different sperm fractions. Because classical sperm-washing procedures have not been shown to remove HPV (*Brossfield et al., 1999*; *Foresta et al., 2011b*), the study also verified whether a well-consolidated washing technique used to remove HIV from spermatozoa (*Savasi et al., 2010*, *2007*; *Semprini et al., 1993*; *Sunderam et al., 2008*; *Zafer et al., 2016*) might also be effective in removing spermatic HPV, and could therefore be routinely employed as clinical practice.

MATERIALS AND METHODS

Fifteen HPV-positive individuals were enrolled in the present study (**TABLE 1**). Enrollment criteria included a healthy body mass index (BMI) of between 18 and 25 kg/m^2, demonstrated fertility (biological fatherhood), a lack of other viral chronic infectious disease, and no fertility-related pathologies or causes of male infertility including varicocele, previous testicular surgery, endocrine disfunction or cancer. *Mycoplasma genitalium*, *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections assessed by urethral swabs were additional exclusion criteria. HPV-positive individuals were clinically identified by the presence of condylomas. DNA isolated from a donor who had never had sexual intercourse was used as negative control. DNA isolated from condylomas was used as positive control.

All samples were previously made anonymous in accordance with the requirements of the Italian Personal Data Protection Code (Legislative Decree No. 196/2003) and the general authorizations issued by the Italian Data Protection Authority. Ethics Committee approval was considered unnecessary because, under Italian law, it is only required in the case of prospective clinical trials of medical products for clinical use (Articles 6 and 9 of Legislative Decree No. 211/2003). All of the patients had given their informed consent, following the Helsinki declaration.

Semen samples were obtained by masturbation after 3–7 days of sexual abstinence, analysed and then washed. All samples were analysed by the same biologist and at the same laboratory. Samples were processed at the laboratory within 2 h of ejaculation according to World Health Organization (WHO) recommendations (*World Health Organization, 2010*). Motility was classified according to the WHO criteria as follows: (i) rapid progressive spermatozoa; (ii) slow progressive spermatozoa; (iii) non-progressive spermatozoa; and (iv) immotile spermatozoa. Sperm concentration was calculated using a Makler chamber. The total sperm count was calculated as: total sperm count ($\times 10^6$/ml) $\times$ ejaculate volume (in ml). The Papanicolaou smear for staining of spermatozoa was adopted for morphological evaluation. Spermatozoa were bacteria-free in all patients, and the leukocyte count was below the WHO threshold (i.e. <1 million/ml).

Sperm-washing consisted of a first step of separation on a 40–80% density gradient (PureCeption kit; Sage; USA) and centrifugation for 30 min at 400*g*. This resulted in four different fractions: motile spermatozoa, round cells, non-motile spermatozoa and seminal plasma. The supernatant was removed, and the sperm pellet recovered and resuspended in 3 ml of fresh medium (Sperm-washing medium; Sage). After a washing step (400*g* for 10 min), 1 ml of medium was gently layered on the pellet, and the tube was incubated at 37°C for 1 h. After swim-up, a supernatant volume of approximately 500 µl was recovered. The procedure has previously been

TABLE 1 SPERM PARAMETERS OBSERVED IN 15 HPV-INFECTED PATIENTS AND A NEGATIVE CONTROL.

ID	Semen volume (ml) (1.5 ml)	pH (≥7.2)	Total spermatozoa (39×10^6)	Spermato-zoa/ ml (15×10^6)	Motile spermatozoa (%) (40%)	Normal morphology (%) (4%)	Non-motile spermatozoa/ ml	Swim-up spermatozoa/ ml	Spermatozoa in pellet/ml
1	1	7	13×10^6	13×10^6	23	1	0.1×10^6	0.05×10^6	3×10^6
2	4.5	7.8	261×10^6	58×10^6	72	8	15×10^6	10×10^6	100×10^6
3	3	8	21×10^6	7×10^6	43	3	0.2×10^6	0.4×10^6	15×10^6
4	1.5	8	126×10^6	84×10^6	52	5	4×10^6	20×10^6	120×10^6
5	2	7.6	72×10^6	36×10^6	50	2	6×10^6	3×10^6	35×10^6
6	3.5	7.8	140×10^6	40×10^6	50	15	3×10^6	12×10^6	50×10^6
7	3	7.8	84×10^6	28×10^6	50	6	2×10^6	5×10^6	40×10^6
8	6	7.4	26×10^6	4.4×10^6	40	3	0.1×10^6	0.01×10^6	6×10^6
9	2	7.8	148×10^6	74×10^6	51	16	5×10^6	13×10^6	50×10^6
10	2	7.8	142×10^6	71×10^6	46	16	12×10^6	10×10^6	30×10^6
11	4.5	7.8	238.5×10^6	53×10^6	45	11	6×10^6	8×10^6	80×10^6
12	6.5	7.8	585×10^6	90×10^6	51	5	10×10^6	10×10^6	50×10^6
13	3.5	8	192×10^6	55×10^6	50	5	15×10^6	0.3×10^6	19×10^6
14	5.5	7.6	187×10^6	34×10^6	38	7	5×10^6	3×10^6	50×10^6
15	2	7.8	52×10^6	26×10^6	35	7	5×10^6	3×10^6	15×10^6
Negative control	2	7.6	140×10^6	52×10^6	50	26	1×10^6	2×10^6	40×10^6

Where applicable, the lower limit of the normal range, as reported by the World Health Organization (*World Health Organization, 2010*), is displayed in brackets in the column headings.

described elsewhere (*Savasi et al., 2007*) and is graphically shown in FIGURE 1. Total DNA was extracted from all five fractions (seminal plasma, cell pellet, round cells, non-motile spermatozoa and motile spermatozoa) resulting from the washing procedure using a DNA purification Maxwell RSC Instrument (Promega; USA) and quantified using a Nanodrop 2000 Instrument (Thermo Scientific; USA). A sample of 1 µg of DNA was analysed by nested PCR. When the DNA concentration was too low for 1 µg to be available, the maximum amount of DNA was analysed, but never less than 100 ng.

PCR was performed employing ReadyMix REDTaq Polymerase (Sigma; USA), and MY09/MY11 (product size 450 bp) and GP5+/GP6+ (product size 150 bp) primers (MY09, CGTCCMARRGGAWACTGATC; MY11, GCMCAGGGWCATAAYAATGG; GP5+, TTTGTTACTGTGGTAGATACTAC; GP6+, GAAAAATAAACTGTAAATCAT ATTC) (*Abreu et al., 2012*; *Bertazzoni et al., 2013*; *Camargo et al., 2011*; *Matah and Sareen, 2012*; *Qu et al., 1997*). These primers anneal to the L1 region of the HPV genome; their sequence is degenerated in order not to be specific for any particular HPV, but rather to be broad spectrum and target the different strains of HPV. As a control, samples were also tested for human 28S rRNA 28S-f, TTAAGGTAGCCAAATGCCTCG; 28S-r, CCTTGGCTGTGGTTTCGC). PCR products were loaded onto 1.5% agarose gel and visualized by ChemiDoc

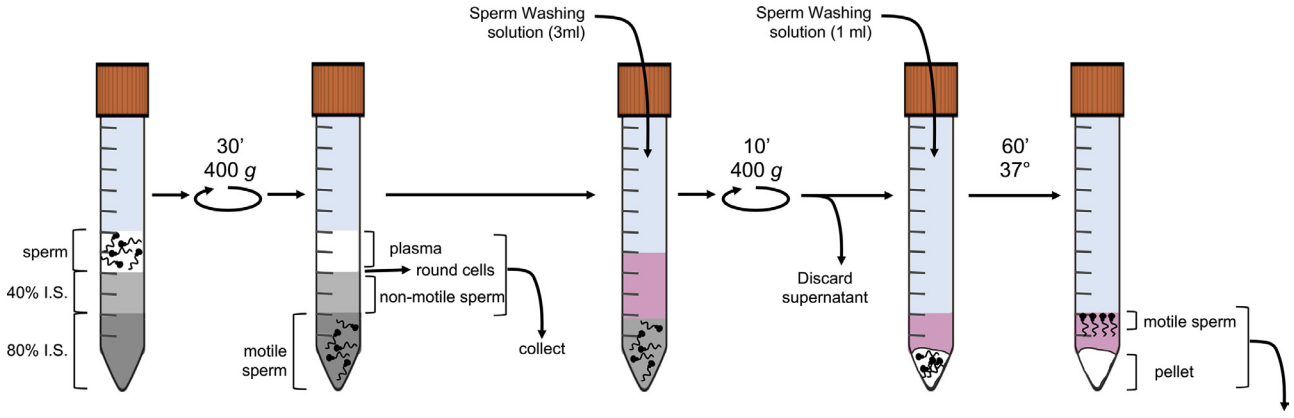

FIGURE 1 Schematic representation of the sperm-washing procedure and of the five fractions – seminal plasma, cell pellets, round cells, non-motile spermatozoa and motile spermatozoa – that were collected for analysis. IS, isotonic solution.

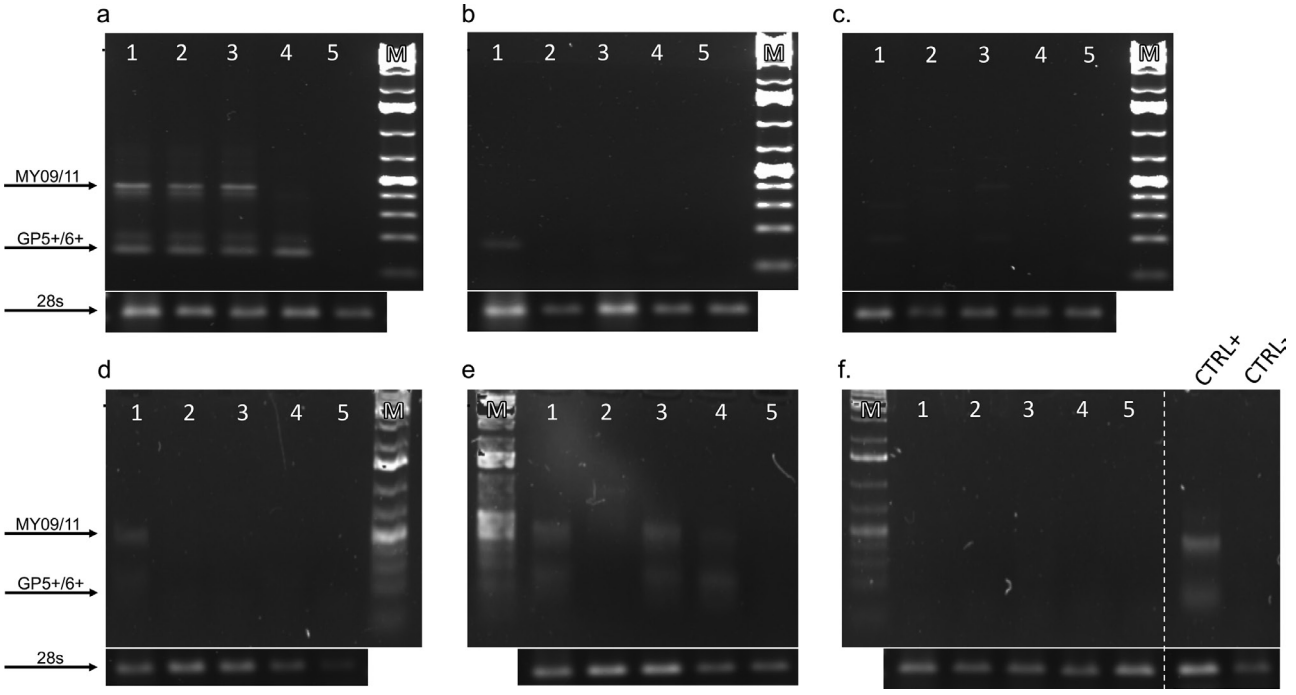

FIGURE 2 Amplification results for human papillomavirus (HPV) DNA, using nested PCR. Each panel shows results obtained from seminal plasma (1), cell pellets (2), round cells (3), non-motile spermatozoa (4) and motile spermatozoa (5), in addition to a 100 bp ladder as a size marker (M). Arrows indicate HPV products amplified by MY09/MY11 and GP5+/GP6+ at 450 and 150 bp, respectively. Representative samples showing HPV DNA positivity in at least one of the fractions are depicted in panels (a) to (e). Panel (f) shows an HPV-negative sample, in addition to a positive and a negative control. For each fraction, the corresponding amplification of the human 28S rRNA is shown at the bottom of each panel.

(Bio-Rad; USA). Along with the PCR product, the 100 bp Plus DNA Ladder (Genespin; Italy) was loaded, in order to have a size marker during the electrophoretic runs. Products from either PCR reaction were cloned into TOPO TA cloning vector (Invitrogen; USA) to determine the limit of detection in the study's experimental condition (*Fenizia et al., 2014*; *Vaccari et al., 2014*). Down to five copies per reaction could be detected, which is consistent with how it has previously been standardized (*Qu et al., 1997*; *Rodrigues et al., 2013*).

RESULTS

Fifteen HPV-positive individuals were enrolled in this study, and their spermatozoa were screened for the presence of HPV DNA by nested PCR. The mean age of the patients was 32.3 ± 7.9 years, and all of them had an adequate BMI. All the five fractions resulting from the sperm-wash procedure described above (FIGURE 1) were analysed for each donor. FIGURE 2 shows the results obtained from six representative individuals, as well as from a positive and a negative control.

HPV DNA was not found in five out of 15 samples (33%) (see FIGURE 2F as an example). Samples obtained from the other 10 donors (67%) gave a positive result for at least one of the fractions, as exemplified in FIGURE 2A–E. No consistent pattern was observed throughout the different samples in the cell pellet, the round cells or the non-motile spermatozoa fractions. Overall, HPV DNA was detected in seminal plasma from 10 patients (67%), in one cell pellet (7%), in five round cell fractions (33%) and in three non-motile spermatozoa fractions (20%), but not in the motile spermatozoa fractions (0%). Notably, whereas HPV DNA was never detected in motile spermatozoa, i.e. in the fraction used for fertilization procedures, after the current sperm-washing procedure, viral DNA was always present in the fraction derived from seminal plasma, indicating that HPV DNA is localized in seminal plasma and not in motile spermatozoa.

Considering that the purpose of this work was to detect the smallest amount of HPV DNA, even the faintest bands, evidence of very few copies, were considered (i.e. FIGURE 2C, lanes 1 and 3). As a control for DNA quality and the presence of potential polymerase inhibitors, PCR was performed on the human gene *28S* as a reference. The results are shown at the bottom of the nested PCR panels for each corresponding sample (FIGURE 2A–F), including positive and negative controls (FIGURE 2F). In each tested fraction, the intended *28S* amplicon was successfully detected.

DISCUSSION

HPV infection is highly prevalent worldwide, but knowledge of the effect of this virus on fertility is still partial at best. Two open controversial questions, in particular, have not yet been unequivocally answered, one regarding the presence of the virus in the spermatozoa and the other regarding the potential influence of HPV infection on seminal parameters.

Recent meta-analyses have shown that HPV semen infection is a risk factor for male infertility (*Foresta et al., 2015*; *Lyu et al., 2017*; *Xiong et al., 2018*). However, the specific mechanism underlying this association has yet to be elucidated. Boeri's observations in his recent study confirmed the potentially detrimental impact of seminal HPV detection on the progressive motility of spermatozoa in a group of infertile men (*Boeri et al., 2019*; *Depuydt et al., 2019*). However, in that

study, the authors evaluated the presence of HPV in the overall spermatozoa, not investigating in depth the different sperm fractions. Capra's experiments were performed using different seminal fractions, similar to the current work. They focused more on identifying HPV genotypes in the different fractions (*Capra et al., 2019*), while the current study used analyses that were as 'pan-HPV' as possible. In fact, the main focus was to obtain HPV-free spermatozoa, suitable for clinical purposes. The results of Capra and colleagues confirm the findings of the present study.

A small group of HPV-infected men were analysed to verify whether, in this situation, HPV could be detected in semen and, if so, which seminal fraction was hosting the virus. Initial results indicated that HPV was present in semen in the majority of the individuals analysed. Analyses of the seminal fraction using a well-known sperm separation procedure showed that the virus was present in the seminal plasma fraction in every HPV-infected semen sample. Notably, HPV could be detected in the cell pellet, round cell and non-motile spermatozoa fractions as well, but was never observed in the motile sperm fraction.

It is still under debate which kind of ejaculated cells HPV can be found in, and whether the virus lies on the cell surface or is located at the intracellular level (*Cortés-Gutiérrez et al., 2017*; *Foresta et al., 2011a*; *Garolla et al., 2012b*, *2012a*; *Pérez-Andino et al., 2009*). Therefore the possible impact of HPV on spermatozoa is unclear, as is the advantage for a virus in infecting spermatozoa, as they are transcriptionally inactive in their mature stage so a virus cannot be reproduced (*Williams and Smith, 1996*). Pérez-Andino suggests that this could be an efficient strategy to bypass female mucosal protection, in order to enhance virus spread and mucosal penetration (*Pérez-Andino et al., 2009*). On the other hand, the observation that HPV was never detected in the motile sperm fraction, the one that fertilizes an egg, implies the prevention of a disastrous fetal infection. Thus, viral infections are transmitted to the unborn child through a maternal infection. If the sperm cell capable of fertilizing an egg had HPV integrated into its DNA, the child would be born with all their cells infected with HPV.

Sperm-washing procedures have previously been tested, but none of them has been able to succesfully and repeatedly remove HPV from the spermatozoa (*Brossfield et al., 1999*; *Foresta et al., 2011b*; *Olatunbosun et al., 2001*; Rintala and Gre, n.d.). This study used a sperm-washing procedure optimized for HIV-infected individuals (*Semprini et al., 1993*) and routinely employed in clinical practice (*Savasi et al., 2007*; *Semprini et al., 1993*; *Sunderam et al., 2008*; *Zafer et al., 2016*). This procedure differs from previous ones (*Brossfield et al., 1999*; *Foresta et al., 2011b*; *Olatunbosun et al., 2001*; Rintala and Gre, n.d.) in that it is based on using multiple approaches, including stratification on density gradient, sperm-wash and swim-up (*Savasi et al., 2010*, *2007*; *Semprini et al., 1993*; *Sunderam et al., 2008*; *Zafer et al., 2016*). Notably, the use of this procedure resulted in a spermatic fraction of motile spermatozoa that was consistently HPV negative in all the analysed samples. In order to analyse such samples, nested PCR was performed, as previously standardized (*Camargo et al., 2011*; *Coutlée et al., 2005*; *Matah and Sareen, 2012*; *Savasi et al., 2010*). This technique is able to detect a minimal amount of specific HPV DNA (down to four copies in our hands) in amounts of 1 µg of DNA per reaction, corresponding to approximately 400 × 10^3 cells. The results confirmed that the motile sperm cell fraction, the one used in fertilization procedures, was always HPV-free.

No consistent pattern of HPV detection was detected in cell pellets, round cells and non-motile spermatozoa. As well as being present within infected cells, HPV can be found attached to the cell surface, can infect cellular debris and can be released from infected cells and be found in plasma (*Foresta et al., 2010b*; *Koutsky, 1997*; *Lowy et al., 1994*). All these caveats notwithstanding, the motile sperm cell fraction was shown to be HPV DNA negative in all the analysed samples, suggesting that the washing procedure used in this study is able to consistently yield an HPV-free seminal fraction.

The present study has some limitations, such as the limited number of analysed samples. However, the vast majority of papers in the literature evaluate the presence of HPV virus in the overall semen, while this study successfully analysed the different fractions. Moreover, the enrolled patients were all demonstrated to be fertile. This differs from most studies, where males with fertility abnormalities have been included.

The present study confirms previous findings that HPV can be detected in semen of HPV-infected men and could be common in men with primary infertility. Some authors have reported that HPV is associated with impaired spermatozoa, in particular with progressive motility (*Boeri et al., 2019*). Overall, these observations reinforce the idea that screening and diagnosis for HPV should be performed in the diagnostic workup of men asking for reproductive assistance, not only because of its potentially negative pathophysiological impact on male fertility, but also to eliminate the virus from the spermatozoa. Thus, as this study has shown that it is possible to obtain HPV-free motile spermatozoa, screening all semen for HPV positivity should be considered in every assisted fertility situation (*Depuydt et al., 2018*).

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