Journal of Medical Virology 81:2007–2011 (2009)

Prevalence of Human Papillomaviruses in Urine Samples of Male Patients Infected With HIV-1 in Sao Paulo, Brazil

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Human papillomavirus is a DNA virus that includes 118 genotypes. HPV16 is responsible for 80% of cervical cancer in women. Men are important reservoirs and major transmitters of HPV to their partners. The aim of this study was to detect HPV DNA and to determine the prevalence of HPV types 6, 11, 16, and 18 in urine samples of men infected with HIV-1. This study included 223 patients infected with HIV-1 from the Center of Reference on HIV/AIDS (CRT-SP) and an outpatient clinic of HIV. Urine samples were collected and after DNA extraction real-time PCR was performed for detection of HPV DNA. Positive samples were then tested by conventional PCR using type-specific primers for the four HPV types. A total of 223 men infected with HIV-1 were tested, 81% of whom were on HAART. Four (5.8%) were positive for HPV6, 18 (26.1%) were positive for HPV11, 22 (31.9%) were positive for HPV16 and five (7.2%) were positive for HPV18 by conventional PCR. Twenty (29%) patients had other HPV types and five patients (1.5%) had multiple types. The mean T CD4+cells count was 517 and 441 cells/mm^3 ($P = 0.30$), in HPV negative and positive men, respectively. The HIV viral load was higher in the HPV negative group than for in the men with HPV ($P = 0.0002$). A 30.9% prevalence of HPV was found in asymptomatic urine samples of men infected with HIV-1. This study suggests that urine may be a useful specimen for HPV screening. ***J. Med. Virol. 81:2007–2011, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: HPV; real-time PCR; men; urine

INTRODUCTION

Human papillomavirus (HPV) genital infection is a common sexually transmitted infection, involving about 50% of sexually active adults [Baay et al., 2004; Scheurer et al., 2005]. Several studies have established the etiologic role of HPV in squamous cell carcinoma of the cervix [Roden et al., 2004] and HPV DNA has been demonstrated in more than 99% of cervical cancer cases and its precursors [Schiffman et al., 2007]. Cervical cancer is the second most common cancer in women with 500,000 new cases and 275,000 deaths yearly worldwide [de Villiers et al., 2004; Bernard, 2005; Pyeon et al., 2005].

The papillomaviruses (PV) are highly diversified [Laimins, 1996; Correnti et al., 2004] and can infect epithelial cells [de Sanjose and Palefsky, 2002]. In the human host, the data suggest the existence of 200 types of HPV, but some 118 HPVs types are described on the basis of isolation and complete genome sequencing of the virus [Laimins, 1996; Correnti et al., 2004; Cox, 2006]. HPV has an icosahedric capside, is non- enveloped and possesses double-strand DNA with about 8,000 bp [Brinkman et al., 2002; Stanczuk et al., 2003; Cox, 2006; Giuliano et al., 2007]. The viral types are classified into two groups according to cancer association: low and high oncogenic risk [Porro et al., 2003].

There are few data about HPV infection in men, but some studies have shown prevalence ranging from 1% to 82.9% [Giovannelli et al., 2007]. The low numbers of diagnosed cases reflect the difficulty in determining appropriate specimens and tests to detect HPV in men [Gravitt et al., 2000]. Major studies have used penis skin—specifically, the coronal sulcus, glans penis, and

Presented in part at the XVII International AIDS Conference, August 3–8, 2008, Mexico City, Mexico.

Grant sponsor: Fapesp; Grant number: 2006/53135-7; Grant sponsor: CAPES.

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Accepted 18 July 2009

DOI 10.1002/jmv.21624

Published online in Wiley InterScience (www.interscience.wiley.com)

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the urethra, due to the direct contact of the penis with the uterine cervix, for determining anatomic sites for detection of HPV [Giuliano et al., 2007].

DNA detection methods using urine specimens have been used to diagnose other sexually transmitted infections. Urine is a potential source of HPV since bladder and urethral epithelia are susceptible to HPV infection [Brinkman et al., 2004]. The aim of this study was to evaluate HPV prevalence in urine samples of asymptomatic men infected with HIV-1 by detection of HPV DNA and to determine the prevalence of HPV types 6, 11, 16, and 18 in HPV DNA positive samples.

MATERIALS AND METHODS

Subjects

Subjects were recruited from two sites: Center of Reference on HIV/AIDS (CRT-SP), which provides care for approximately 3,000 subjects infected with HIV-1, from where 148 men were invited at random when they were seen on a screening visit for conditions such as prostate cancer or sexual impotence. Additionally, 75 men infected with HIV-1 from our outpatient HIV clinic (Adee3002/HCFMUSP) in a general hospital in Sao Paulo were also randomly included. The sample size was calculated based on an estimated prevalence of 20% and a confidence level of 95%, using Epi Info v.6.04 (Centers for Disease Control and Prevention, USA, 2001). This study was approved by the Ethical Research Board of both institutions, namely CRT-SP and Hospital das Clínicas, Sao Paulo University Medical School (HC/FMUSP). After signing the informed consent, subjects were requested to answer a standardized interview including questions on ethnic background, age, educational level, sexually transmitted diseases (STD) history, and sexual behavior. The subjects were also submitted to a urologic examination. The HIV plasma viral load, CD4+ T cells count and time of first HIV serology were all obtained from hospital records. Samples consisting of 60 ml of urine were collected from the 223 men infected with HIV-1 included on the study. Patients who presented any visible lesion or who complained of current symptoms suggestive of an STD were not included in the analysis.

DNA Extraction

Urine samples were stored at −70°C until DNA extraction, using a commercially available kit (GFX Genomic Blood DNA Purification Kit, Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

PCR for Detecting HPV DNA

The DNA extractions were amplified using the oligonucleotides PGMY9 and PGMY11 [Gravitt et al., 2000] which amplify a conserved region of 450 bp of L1 gene of several types of HPV, adapted to the real-time PCR technique using the SYBR Green®. For 20 μl of reaction mix, a 10 pmol mixture of primers PGMY9/PGMY11 and SYBR® GreenER qPCR Supermix Universal Buffer (Invitrogen, Carlsbad, CA) was used. Negative and positive controls were included in all reactions. The positive control was a sample donated by the Laboratory of Virology of Institute of Tropical Medicine of Sao Paulo confirming the presence of HPV DNA and the negative control was a reaction mixture without template DNA. An initial step of 4 min at 95°C for DNA denaturation was followed by 45 cycles of 1 min of denaturation at 95°C, 1 min of annealing at 58°C, 1 min at 72°C for extension, and a last step of 7 min at 72°C for final extension. The melt curve was conducted to detect primer dimers and to analyze reaction specificity, with an initial temperature of 60°C + 0.5°C/cycle for 30 sec.

Type-Specific PCR

To confirm the presence of HPV 6, 11, 16, and 18 in HPV DNA positive samples by real-time PCR, a conventional PCR was carried out using type-specific primers [Naqvi et al., 2004] to the four types. The PCR was conducted with 25 μl of reaction mix containing 0.2 mM dNTPs, 2 mM MgCl2, 10 pmol each primer and 0.5 U Taq DNA polimerase (Amersham Pharmacia Biotech, Piscataway, NJ) and 1× PCR buffer (10 mM Tris–HCl and 50 mM KCl). Negative and positive controls were included in all reactions. One positive control was used for each HPV type (6, 11, 16, and 18) at each reaction. The positive controls were also kindly donated by the Laboratory of Virology of Institute of Tropical Medicine of Sao Paulo. The PCR products were submitted to electrophoresis of agarose gel 2% under the following conditions: 100 V, 400 mA, and 60 min. Visualization was made possible by the addition of ethidium bromide under UV light. The results were interpreted by following product sizes: HPV 6–280 bp; HPV 11–360 bp; HPV 16–217 bp, and HPV 18–700 bp [Yamazaki et al., 2001; Naqvi et al., 2004].

Statistical Analysis

Statistical analyses were performed with the aid of Epi Info v.6.04 (Centers for Disease Control, USA, 2001). Significance analyses were performed employing the χ^2 test with Yates correction or Kruskal–Wallis nonparametric test for continuous variables.

RESULTS

HPV DNA was detected in 69 (30.9%) patients. Four (5.8%) patients were positive for HPV 6, 18 (26.1%) were typed as HPV 11, 22 (31.9%) as HPV 16, and 5 (7.2%) as HPV 18. Twenty men had demonstrable HPV DNA but did not react with any of the four type-specific primers used. In addition, five patients were co-infected with more than one type of the four types of HPV mentioned above. One patient had triple infection with HPV 6, 16, and 18 (Fig. 1).

Table I displays the age and sexual behavior of patients. HIV/HPV co-infected men and HPV-negative men had similarly precocious beginning of sexual

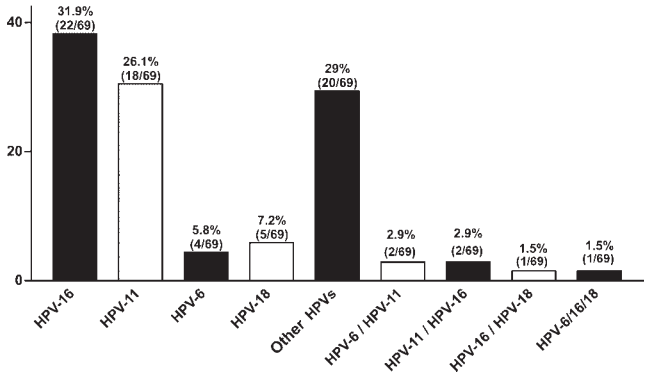

Fig. 1. Prevalence of HPV types in urine samples from men infected with HIV-1.

activity, with a mean age of 14 years or less (33.4% vs. 40.3%, $P = 0.22$). The number of sexual partners was also similar for both groups of patients over the last year [40.6% vs. 36.4%, $P = 0.50$ (data not shown)]. Lack of condom use during oral sex was a risk factor for HPV transmission ($P = 0.004$).

Mean HIV RNA plasma viral loads were 10,663 copies/ml and 14104 copies/ml, for HPV positive and HPV negative individuals, respectively ($P = 0.0002$), while mean T CD4+ cells counts were 441 and 517 cells/mm^3, respectively, for HPV positive and HPV negative patients ($P = 0.30$) (Table II). Thus, although HIV viral load was significantly lower for HPV positive patients, no statistically significant difference was found in CD4 counts between the 69 co-infected HPV/HIV patients and patients infected with HIV only.

DISCUSSION

This study found a prevalence of 30.9% of HPV DNA in urine of men infected with HIV-1, similar to that found in several studies employing conventional diagnostic tools [Geddy et al., 1993; Kjaer et al., 2000; Aynaud et al., 2003; Winer et al., 2003; Brinkman et al., 2004; Golijow et al., 2005; Giovannelli et al., 2007; Daponte et al., 2008; Kreuter et al., 2008; Yamada et al., 2008]. Strauss et al. [1999] found HPV DNA in 78% and 65% of cervical samples and urine, respectively, with 76% parity between the two samples. Sellors et al. [2000] showed the presence of HPV DNA in urine samples of 45% of their patients, and at least one type of high-risk oncogenic HPV in cervical or urine samples from 69% of women tested. The rates of HPV infection in men present a broad range, due to different profiles of patients, different tests, and different clinical samples, such as penile surface, foreskin cavity, glans penis, scrotum, urethra, semen and urine. These difficulties are due to a lack of consensus regarding the ideal clinical specimen [Giovannelli et al., 2007]. Even without any recognizable clinical lesion, the male urethra is the major reservoir of HPV, which can explain the relapse after anti-HPV treatment of the sexual partner [Aynaud et al., 2003].

Tests for HPV DNA in urine samples could also be employed in preliminary screenings for cervical cancer and as additional testing to Pap smear for women. Indeed, in another study, HPV DNA was detected in the urine of more than 70% of women with cervical cancer [Song et al., 2007]. Cervical samples and urine were used to detect oncogenic HPV types from women with different stages of lesions. HPV prevalence of 48.1% and 33.8% was found among women with pre-malignant or malignant lesion by histology and urine samples, respectively, with an overall urine HPV detection sensitivity of 70% [Daponte et al., 2006]. It has been suggested that urine samples were reliable to screening HPV in women with different stages of cervical disease including cancer [Daponte et al., 2006]. These data suggest that urine samples are particularly useful for the detection of HPV, and that further studies using urine should be conducted in order to compare urine with more established HPV detection methods. Urine collection is simple and can provide a convenient, non-invasive specimen for the screening of HPV infection [Geddy et al., 1993].

TABLE I. Age and Sexual Behavior of Patients

Characteristics	HPV		P value
	Positive (n = 69)	Negative (n = 154)	
Age (years)			
18–29	3 (4.3%)	4 (2.6%)	0.61
30–39	13 (18.8%)	36 (23.4%)	
≥40	53 (76.9%)	114 (74%)	
Age of the first sexual relation			
≤14 years-old	23 (33.4%)	62 (40.3%)	0.22
≥15 years-old	39 (56.5%)	85 (55.2%)	
Not answered	7 (10.1%)	7 (4.5%)	
Use of condom during active oral relations			
No practice	30 (43.5%)	59 (38.3%)	0.004
Always	6 (8.7%)	21 (13.6%)	
Sometimes	16 (23.2%)	24 (15.6%)	
Never	13 (18.8%)	50 (32.5%)	
No answer	4 (5.8%)	0 (0%)	

TABLE II. Clinical and Laboratorial Characteristics of the Patients

Characteristics	HPV		*P* value
	Positive (n = 69)	Negative (n = 154)	
Genital warts (if they had at some point in their lives)			
Yes	25 (36.2%)	42 (27.3%)	0.14
No	37 (53.6%)	103 (66.9%)	
Not Answer	7 (10.2%)	9 (5.8%)	
Wounds (if they had at some point in their lives)			
Yes	9 (13%)	42 (27.3%)	0.06
No	53 (76.8%)	100 (64.9%)	
No answer	7 (10.2%)	12 (7.8%)	
Use of HAART			
Yes	60 (87%)	115 (74.7%)	0.03
No	6 (8.7%)	35 (22.7%)	
No answer	3 (4.3%)	4 (2.6%)	
Current HIV viral load (copies/ml)			
≥1,000	25 (36.2%)	91 (59.1%)	0.0002
<1,000	39 (56.5%)	43 (27.9%)	
No data	5 (7.3%)	20 (13%)	
Current T CD4+ cells count (cells/mm^3)			
<200	13 (18.8%)	19 (12.3%)	0.30
≥200	51 (73.9%)	117 (76%)	
No data	5 (7.3%)	18 (11.7%)	

HPV 16 was the most prevalent type found in this study, as previously found in other studies [Stanczuk et al., 2003; Gupta et al., 2006; Song et al., 2007]. In fact, HPV 16 was present in 59% and 61% of cervical and urine samples, respectively, all coming from patients who had invasive squamous cell carcinomas [Stanczuk et al., 2003]. Brinkman et al. [2002] showed similar rates of detection of HPV DNA, 10.2% and 10.4%, in urine samples and cervical swab specimens, respectively, of women infected with HIV-1. Thus, there is a high prevalence of HPV types in urine of subjects infected with HIV-1, with a huge type diversity and co-infection with multiple types.

This study showed that the lack of condoms use during oral sex was a risk factor for HPV acquisition. Several studies have found that condom use by men does not reduce the risk of HPV infection in women [Kjaer et al., 2000; Winer et al., 2003]. Although condom use decreased the risk of HPV acquisition, even among men who reported using condoms at every intercourse, acquisition of HPV could not be entirely avoided. The potentially protective effect of condoms is controversial for both men and women. Currently, there is a concept that condom use provides effective protection against several other sexually transmitted diseases like HIV and *Chlamydia*, but that they are not effective for preventing HPV infection. The fact that condoms are often not used during the entire sexual intercourse and the multifocal nature of HPV infection in women make the limited protective effect of condom use in relation to HPV acquisition in men understandable [Kjaer et al., 2000], confirming our data, since men who reported using condoms sometimes during oral sex were at heightened risk for acquiring the HPV.

Immunosupression may be an important factor for HPV persistence. The absolute number of T CD4+ cells in patients infected with HPV was lower than in the non-infected group in our study, but the difference did not reach statistical significance. In contrast, the number of T CD4+ cells was lower in patients with penile intra-epithelial neoplasms compared with the whole cohort, in a study from Germany [Kreuter et al., 2008], and in a Kenyan study a lower number of T CD4+ cells and a significantly higher HIV-1 viremia was found in women who were co-infected with HPV [Yamada et al., 2008]. Thus, the use of HAART in our patients may contribute to improvement of T-cell immunity and lower viral loads of HIV.

This study was the first to detect HPV DNA in urine by real-time PCR in Brazil, and a high prevalence of HPV was found in asymptomatic patients with no history of HPV lesions. In a previous study, real-time PCR showed higher sensitivity when compared with conventional PCR [Daponte et al., 2008]. It is believed that the asymptomatic nature of HPV infection in men is responsible for the high rate of transmission to their partners [Golijow et al., 2005]. Thus, screening of men for HPV becomes relevant to the prevention of cervical cancer in women [Giovannelli et al., 2007]. In this study real-time PCR was a useful and rapid tool for HPV detection in urine samples. Since urine is an easy, non-invasive specimen to obtain, a higher compliance with HPV screening among men and women alike seems likely.

ACKNOWLEDGMENTS

We thank all patients who participated in this study, as well as Dr. Dana Gallo for critical revision of this manuscript and Dr. José Eduardo Levi for providing the positive controls.

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