Human papillomavirus infection in multiple sites

Infecção por papilomavírus humano em múltiplos sítios

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ABSTRACT

Introduction: Studies concerning human papillomavirus natural history have been focused on cervical infection and disease, but have scarcely described anal infections, especially in clinically health population. Hence, knowledge on HPV natural history is recently being investigated although viral maintenance in hosts is poorly understood. Detection on diverse, extragenital sites may add in the elucidation of infection-dissemination-reinfection cycle reported on the human genital tract. Besides that, it is evident the importance of an adequate screening of the viruses for appropriate diagnosis, mainly in the first steps of the neoplasia in order to provide a better prognosis. Our results pointed out HPV prevalence rates in genital lesions of 87% (80/92) and of 47.8% (44/92) for anal samples. We pointed out that this high prevalence is due to clinically detected lesions, that contributes to the risk of extragenital infections, mainly by host auto-inoculation. Despite the difference in samples size, we did not find statistical relevant differences related with genera (men 85.1% and women 92%; p>0.05), corroborating the idea of no tropism differences by sex. Nevertheless, for anal samples, statistically significant differences were found between men and women (68% of anal HPV infection in women against 40.3% in men (p=0.038). These results reveal that female anal infections are more frequent than in men, suggesting that infection by autoinoculation occurs and can render this site as an HPV reservoir, occasionally becoming clinical lesions. Hormonal profile, sexual behavior and differences among body sites can explain these differences here described. Our study is a preliminary evaluation of HPV infection in human multiple sites.

Keywords: papillomavirus infections; polymerase chain reaction; anus.

RESUMO

Introdução: Muitos estudos sobre a história natural do papilomavírus humano (HPV) têm focado nas infecções cervicais, mas pouco tem se falado sobre infecções anais em pacientes assintomáticos. Assim, a história natural do HPV ainda está em construção e os mecanismos de manutenção dele no organismo hospedeiro são pouco compreendidos. Entendemos que a detecção do vírus em sítios extragenitais poderá ajudar na compreensão da cadeia de infecção-disseminação-reinfecção pelo HPV no trato genital humano. Além disso, é evidente a importância de um rastreio adequado do vírus diante de diagnósticos clínicos, principalmente no período inicial da lesão, provendo, assim, um melhor prognóstico. Nossos resultados apontaram uma prevalência da infecção por HPV em lesões genitais de 87% (80/92) e nas amostras anais tal prevalência foi de 47,8% (44/92). Ressaltamos que nossa prevalência foi alta, mas estudamos indivíduos com lesões genitais clinicamente detectadas, aumentando, assim, o risco de infecção em outros sítios e confirmando a ocorrência da presença do HPV no hospedeiro, provavelmente decorrente de autoinoculação. Apesar da diferença entre o número de indivíduos dos sexos feminino e masculino em nosso estudo, não encontramos diferenças estatísticas relacionadas à presença do HPV em lesões genitais (H: 85,1% e M: 92%; p>0.05), corroborando que não há diferenças entre o tropismo por gênero referente às infecções genitais. Entretanto, no caso das infecções anais, a porcentagem foi estatisticamente diferente: 68% de HPV anal em mulheres e 40,3% em homens (p=0,038). Estes resultados apontam que a aquisição da infecção anal em mulheres, apesar de assintomática, é mais frequente do que em homens, sugerindo que esta mucosa possa ser infectada por autoinoculação e funcionar como um reservatório feminino, o que eventualmente poderá resultar em lesões. Tais discrepâncias da prevalência entre homens e mulheres poderiam ser explicadas pelo comportamento sexual, por efeitos hormonais e por diferenças entre sítios. Nosso estudo se trata de um levantamento preliminar da infecção por HPV em múltiplos sítios.

Palavras-chave: infecções por papilomavírus; PCR; ânus.

INTRODUCTION

The genital infection caused by the human papillomavirus (HPV) is a sexually transmitted disease (STD) that affects about 50% sexually active population[1]. HPV infections contribute to about 5.2% human cancers in the world population, including anal, genital, and oropharynx cancers[2,3]. HPV infection is the most common STD in the world. In the United States, approximately 14,000 people are infected with human immunodeficiency virus every year and 79,000 have persistent infection, whereas the majority of sexually active persons will detect infection by the virus at least once in a lifetime[4]. In Brazil, cervical cancer is the third most common type of cancer affecting women: about 15,590 new cases of cervical cancer were reported in 2014. Anal cancer ranks second among the most common types of cancer caused by HPV[5]. This neoplasm is relatively rare compared with cervical cancer in the population, with incidence of 1.5 per 100,000 people[6].

The incidence of anal cancer has been growing, and this type affects both men and women. However, in the general population, anal cancer is more common among women than among men[7]. Similar to cervical cancer, anal cancer is preceded by a series of pre-cancerous changes, that is, several degrees of anal intraepithelial neoplasia, raising the possibility that it can be avoided if detected early.

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The prevalence of HPV in different countries was nearly 100% in patients with cervical cancer; 40% in cases of penile, vulva, and vagina cancers; 90% in cases of anal cancer; 3% in cases of oral cancer; and 12% in cases of oropharyngeal cancer[8].

Cervical cancer is still the second leading cause of death by cancer among women[1]. The incidence of anal cancer continues to grow, and the presence of HPV in such cases is around 90%[7,9]. While it is already established that HPV is the causative agent of cervical cancer and anal carcinoma, little is known about the etiology of oral and even penile cancer[10]; however, it is accepted that HPV is a predisposing factor, along with alcoholism and smoking.

Many studies of natural history of HPV have focused on cervical infections, but little has been stated about anal infections in asymptomatic patients. Thus, the natural history of HPV is still under construction and its maintenance mechanisms in the host organism are little understood. We understand that the detection of the virus in extragenital sites could help in understanding the chain of infection-spread re-infection by HPV in the human genital tract. Furthermore, the importance of a proper screening of the viruses before clinical diagnosis is clear, especially in the initial period of the lesion, providing thus a better prognosis.

The objective of this study was to contribute to the knowledge about the natural history of HPV infection, investigating whether genital infection predisposes to anal infection by determining the presence of HPV infection in both sites. In addition, this study had the big purpose of confirming the diagnosis of genital HPV by polymerase chain reaction (PCR) in genital samples with lesion suggestive of HPV paired with anal mucosa samples.

METHODS

This was a cross-sectional study comprising 92 patients, totaling 184 samples, conducted in the Virological Diagnostic Laboratory of the Universidade Federal Fluminense (UFF). The samples were collected between 2009 and 2014 in the Sector of Sexually Transmitted Diseases of UFF and at Santa Casa de Misericordia of Rio de Janeiro. Two samples were taken from each patient: genital and anal. Genital samples were biopsies of benign lesions, whereas anal samples were only healthy mucosa smears.

The samples were collected in Tris-EDTA buffer (pH 7.2) and frozen at -20°C in the Sector of Sexually Transmitted Diseases of UFF and the Sector of Dermatology of Santa Casa. They were later transported to the Virological Diagnostic Laboratory of UFF and kept at -20°C.

DNA extraction

The method used was phenol-chloroform extraction. For inactivation of possible pathogens in the samples, the tubes were placed in a water bath at 56°C for 2 hours. Next, 100 µL digestion buffer was added (50 mM Tris-HCl (pH 8.5), 10 mM EDTA, 200 µg/mL proteinase K) in a water bath at 55°C for 3 hours or 37°C overnight. Continuing to extraction, phenol–chloroform method was used, starting with the addition of 1 mL phenol/chloroform/isoamyl alcohol (25:24:1; Lifetech) in each tube, containing around 500 µL sample.

The sample was homogenized for 5 minutes, and then centrifuged for 15 minutes at 8,000 rpm. Two layers were formed in the tube, from which the supernatant is transferred to a new Eppendorf tube. If the top aqueous layer was not clear, centrifugation was repeated. All procedures were performed by the hood and the residues were appropriately discarded in chemical waste.

Samples were mixed with a solution in the ratio 1:10 of 3 M sodium acetate (pH 6.0), then with absolute ethanol in the proportion of 2.5 times the volume of the aqueous layer. After mixing by inversion, the samples were frozen at -20°C overnight.

In the following phase, specimens underwent a 30-minute centrifugation at 14,000 rpm at 4°C. Then, the supernatant was discarded, 70% ethanol was used for washing it, and another 15-minute centrifugation at 14,000 rpm was performed. After discarding the 70% ethanol, the tubes went through drying process, for complete evaporation of the ethanol in a thermoblock at 60°C for 2 hours. Finally, the DNA adhered to the tube was resuspended in 50 mL distilled water and the samples were then stored at -20ºC.

Generic polymerase chain reaction with consensual primers MY09/MY11

Generic PCR allows detection of any HPV genotype using consensual primers MY09/MY11, which amplify a 450-pb fragment corresponding to L1 gene[11].

The reaction mixture was composed of 5 mL of 10´ PCR buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM NaCl), 1 mL dNTP (200 mmol dATP, dCTP, dGTP, dTTP), 3 mL $MgCl_2$ (50 mM), 1 mL primer (50 pM) to 0.25 mL DNA Taq polymerase, totaling 50 µL reaction with the addition of the sample. We used primers for the human actin gene (Ac1 and Ac2) as DNA extract control, positive samples as positive control, and ultrapure water as negative control. Samples were subjected to 40 amplification cycles: 94°C for 1 minute (denaturation), 55°C for 1 minute (hybridization), and 72°C for 1 minute (extension). At the end of cycles, the stabilization phase was of 10 minutes at 72°C.

Polymerase chain reaction with internal primers GP5+/GP6+

For confirmation of positive or negative samples, the nested PCR was performed using primers GP5+/GP6+ (150 bp). The reaction mixture was composed of 5 µL of 10´ PCR buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM NaCl), 1 mL dNTP (200 mmol dATP, dCTP, dGTP , dTTP), 3 mL $MgCl_2$ (50 mM), 1 mL primers (50 pM), and 0.25 mL DNA Taq polymerase, totaling 50 µL reaction mix with addition of 2 mL MY amplicon performed in the previous step.

The amplification reaction consisted of 35 cycles as follows: 94ºC for 4 minutes (pre-denaturation), 94°C for 30 seconds (denaturation), 45°C for 1 minute (hybridization), and 72°C for 1 minute (extension). Finally, samples were put at 72°C for 10 minutes (stabilization).

Polymerase chain reaction with specific primers

In this step, samples considered positive by a generic PCR amplification were submitted to specific PCR for HPV genotyping. The primers used were specific for the E6/E7 segment of HPV types 6, 11, 16, 18, 33, and 45 (synthesized by Invitrogen®), resulting in 89-230 bp. As a positive control for HPV types 16 and 18, DNA from HeLa and CaSki cell lines, respectively, were used. For other types, positive samples were used, and sterile distilled water was used as a negative control.

For the reaction to occur, a mixture was prepared containing 5 µL of 10´ buffer solution (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM NaCl), 1 µL of dNTP (200 µmol dATP, dCTP, dGTP, dTTP), 3 µL $MgCl_2$ (50 mM), 1 µL primer (50 pM), and 0.25 Platinum DNA Taq polymerase.

The amplification reaction consisted of 35 cycles as follows: 5 minutes at 94°C (pre-denaturation), 30 seconds at 94°C (denaturation), 30 seconds at 55°C (hybridization), and 1 minute at 72°C (extension). Finally, samples were left for 10 minutes at 72°C (stabilization).

Typing of human papillomavirus by restriction fragment length polymorphism

This technique was used for samples positive for generic HPV PCR by negative in specific PCR, which were . Restriction fragment length polymorphism (RFLP) uses PCR products of the L1 gene, that is, the generic PCR products with consensual primers MY09/MY11. Amplified DNA (3 µL) was added to 2 units of endonucleases *Bam*HI, *Dde*I, *Hae*III, *Hin*fI, *Pst*I, and *Rsa*I (10 U/µL–2 µL; Invitrogen®) and placed separately in 200 µL tubes for digestion. To maintain optimum enzymatic conditions, 1 µL buffer corresponding to each enzyme was added, as well as 4 µL TE buffer (0.5 M Tris (pH 7.4) and 0.5 M EDTA (pH 8.0)), totaling 10 µL in volume of reaction mix in each Eppendorf tube.

After the addition of all components, the enzymatic digestion proceeded at 37°C for 2 hours.

Results of pcr and restriction fragment length polymorphism

PCR results were revealed by electrophoresis in 1.5% agarose gel (Invitrogen®). The amplified products were stained with 2 µL xylene cyanol/bromophenol blue, using standard molecular weight phage λ of 100 bp (Invitrogen®). Then, the gel was stained with ethidium bromide, and the products were visualized on an ultraviolet transilluminator.

For disclosure of the RFLP technique, the total volume of sample (10 µL) was added to 2 µL xylene cyanol/bromophenol blue dye applied to 1.5% agarose gel (Invitrogen®), with the standard molecular weight (50 bp). The products of this digestion were subjected to electrophoresis for about 2 hours. After the run, the gel was stained with ethidium bromide solution. The fragments generated by digestion were visualized on an ultraviolet transilluminator. The samples were identified and typed according to the procedures by Melgaço et al.[11].

Statistical Analysis

Data of each patient and respective results were included in a database. Statistical analysis was performed using the features available in Epi Info software, version 6.2, and Fisher's exact test was applied to build tables with 2 ´ 2 comparative analysis.

RESULTS

This study consisted of 184 samples from 92 patients. We found 67.3% (124/184) positive and 48.3% (60/184) negative samples for HPV.

In 87% (80/92) genital samples, DNA of the virus was found, with a prevalence of 47.8% (44/92) in anal samples. Only 12 (13%) samples resulted in no genital lesion by the virus, whereas 48 anal samples (52.2%) were negative for HPV.

Regarding the prevalence of HPV according to gender, we found 25 (50 samples) female patients and 67 (134 samples) male patients positive for HPV. Among 50 female samples, HPV infection was found in 40 (80%). Among these 40 samples positive for HPV, 23 (57.5%) were genital lesions and 17 (42.5%) were anal mucosa material. Among 10 samples negative for HPV, 2 (20%) were genital samples and 8 (80%) were anal mucosa material. As for males, among the 134 samples studied, 84 (62.6%) had HPV and 50 (37.3%) did not. Of the total 84 samples positive for HPV, 57 (67.8%) were genital lesions and 27 (32.1%) were from the anal mucosa. As for the 50 negative samples, 10 (20%) were related to genital lesions and 40 (80%) to anal smears.

In the presence of viral types, low risk was found in 92.6% (76/82) infections (excluding mixed infections) and high risk in 7.3% (6/82). In viral typing, the most common genotype among the tested types was HPV 11 (43/82), followed by HPV6 (33/82), HPV18 (3/82), HPV16 (2/82), and HPV45 (1/82). Mixed infections were found in 37 samples of HPV 6:11; 1 sample of HPV 6:16; and 1 sample each of HPV types 6, 11, 16, 18, and 45.

Genital concurrence in two sites was found in 10 cases: 3 of HPV6, 5 of HPV11, and 2 of HPV6 and HPV11.

DISCUSSION

In Brazil, the incidence of the most common malignant diseases due to HPV, cervical cancer, is still high. A survey conducted by the Brazilian National Cancer Institute (INCA) showed about 17,000 new cases of cervical cancer per year. In 2013, about 4,160 women died from the disease. In 2015, about 16,000 new cases are expected to rise[12].

As for men, the natural history of infection is little understood. It is known that penile cancer may have HPV DNA present in 30 to 80% of cases[13-16].

Epidemiological estimates of the World Health Organization (WHO)[17] suggest that the prevalence of HPV infection worldwide is between 9 and 13%, equivalent to 630 million people. In genital lesions, this prevalence can reach 100%. This study showed positivity of 67.3% among 184 samples analyzed, as among genital lesions 87% were positive for HPV, which is close to the literature[18].

In the anal site without clinical lesions, 47.8% samples were positive (**Table 1**). In literature, these data vary widely and few studies have been published. The prevalence of HPV in anal site in heterosexual populations is very heterogeneous in these papers, ranging from 1.2, 8, and 24.8%[19-21]. We emphasize that the prevalence we found is high, especially for HPV types 6 and 11 (**Table 2**), but we studied subjects with genital lesions clinically detected, thus increasing the risk of infection in other sites and confirming the presence of HPV in the host probably due to self-inoculation.

Despite the difference between the number of female and male individuals in this study, we found statistical differences as to HPV in genital lesions (males, 85.1%; females, 92%; p>0.05), confirming that there is no difference between tropism by gender in genital infections. However, in the case of anal infections, percentages were statistically different: 68% anal HPV in women and 40.3% in men (p=0.038). These results suggest that the acquisition of anal infection in women, though asymptomatic, is more frequent than that in men, suggesting that the mucosa may be infected by autoinoculation and function as reservoir that may eventually result in injury in women[22]. Such discrepancies in the prevalence among men and women could be explained by sexual behavior, hormonal effects, and differences between these sites.

This study is a preliminary survey of HPV infection at multiple sites. The research is currently in progress, aiming to increase our sample and assess the natural history of HPV.

Table 1 – Prevalence HPV DNA by polymerase chain reaction in genital lesions and anal site.

Samples by site of infection	HPV infection		
	Males n (%)	Females n (%)	Prevalência total n (%)
Genital	57/67 (85.1)	23/25 (92)	80/92 (87)
Anal	27/67 (40.3)	17/25 (68)	44/92 (47.8)

HPV: human papillomavirus.

Table 2 – Human papillomavirus genotypes detected by polymerase chain reaction and specific restriction fragment length polymorphism in genital lesions and anal site.

Samples	HPV genotypes					
	HPV6	HPV11	HPV16	HPV18	HPV45	Mixed infections
Genital	20	30	2	1	1	26
Anal	13	13	–	2	–	13

HPV: human papillomavirus.
*In three anal samples, HPV typology could not be identified.

Conflict of interests

The authors report no conflict of interests.

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