High prevalence of human papillomavirus 16 in penile carcinoma

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Summary. In order to analyze the incidence and prevalence of Human Papillomavirus (HPV) in penile carcinoma, we studied 49 patients with penile carcinoma. Formalin-fixed, paraffin-embedded tissue samples were collected from 64 samples of penile carcinoma from the Hospital General Universitario (Albacete, Spain). Cases were histologically classified and the polymerase chain reaction (PCR) method was used to detect the presence of HPV. Two sets of consensus primers were used, the My09/My11, and the GP5+/GP6+. All positive cases were sequenced in order to establish the implicated genotype. Our results showed that 38 of the 49 cases were positive for HPV (77,5%). HPV16 appeared in 32 (84,2 %) of the 38 positive cases and HPV18 in 4 (10,5%). Our data demonstrate that the My09/My11 primers are more sensitive than GP5+/GP6+ primers, although the combination of the two sets of primers notably increased the total number of HPV positive cases detected.

Key words: HPV, Penis, Carcinoma, PCR

Introduction

Penile carcinoma is an uncommon tumor that has a low yearly incidence in Europe and North America, of 0.1-0.9/100,000 inhabitants (Ferreux et al., 2003). In some regions of Africa, Asia and South America, the prevalence of this tumor increases and may represent 10 to 20 % of all malignant tumors in males (Malek et al., 1993), with a yearly incidence of 4-4.5/100,000 inhabitants (Wabinga et al., 2000). Penile carcinomas are tumors that appear in the adult population with peak incidence around the sixth decade of life (Soto et al.,

2003).

Several risk factors for the development of penile carcinomas have been identified. These carcinomas are more frequent in uncircumcised males, in patients with phimosis (Castellsagué et al., 2002), and in males with multiple sexual partners (Hung-Fu et al., 2001). Immunodeficiency may influence the course of the neoplasm (Poblet et al., 1999).

Human papillomavirus (HPV) infection appears to play an important role in the development of penile carcinomas. More than 200 genotypes of papillomavirus have been described, and about 100 have been found in humans (Bernard, 2005). The sexually transmitted HPV are part of the A subgroup, also known as alpha-papillomaviruses, and represent around 30 genotypes (Doorbar et al., 2005). In the uterine cervix, HPV has been demonstrated to be an important etiopathogenic factor in the development of preneoplastic and neoplastic lesions. It is considered that almost 100% of cervical carcinomas are HPV related, and the role of HPV in malignant transformation of the cervical epithelium has been well established (Bosch et al.,1995; van Muyden et al., 1999). Genital HPVs are classified into low-risk and high-risk types on the basis of their association with preneoplastic or neoplastic lesions. Low-risk HPVs, such as HPV types 6 or 11, are usually associated with benign verrucous lesions, or with low grade intraepithelial lesions that rarely progress to cervical carcinomas. High-risk HPVs, such as HPV types 16 or 18, are usually found in high grade intraepithelial lesions or cervical carcinomas (zur Haunsen, 1996; Villa, 1997). The oncogenic role that high risk HPVs play is associated with the expression of E6 and E7 proteins that interfere with the functions of the p53 and pRB, respectively (Münger et al., 2004).

According to Cubilla et al. (2001), penile carcinoma can be classified into several types: papillary, basaloid, warty, verrucous, sarcomatoid, and usual types. Warty and basaloid carcinomas have been found to show a

strong association with HPV, and an etiological relationship between these types of tumors and HPV is probable. However, in the other types of penile neoplasias, the etiological association with HPV is questionable (Ferreux et al., 2003).

Polymerase chain reaction (PCR) is the standard method for detection of HPV in clinical samples from patients suspected of being infected with HPV. The use of a single primer for detection of HPV in cervical carcinoma has been shown to underestimate the number of cases in which HPV is present; therefore, the use of at least two different primers to avoid this problem is usually recommended (Karlsen et al., 1996). In the present study, two different sets of consensus primers to detect HPV DNA in penile carcinomas were used, an approach that, to the best of our knowledge, has not been previously undertaken. In addition, all positive cases were sequenced to detect the HPV genotype implicated, and correlations between detected genotypes and histological subtypes of penile carcinoma were established.

Materials and methods

One hundred formalin-fixed and paraffin-embedded tissue specimens from patients attended at the Hospital General Universitario of Albacete from 1993 to 2003, were included in the study. The specimens were collected from the archives of the Pathology Department and corresponded to malignant penile lesions or lesions suspected of malignancy. From each case, paraffin blocks and routinely stained haematoxylin and eosin slides were retrieved. All cases were reviewed and diagnostic groups were assigned and graded according to standard histological criteria (Cubilla et al., 2001). From each paraffin block, three 5-μm thick sections were placed in sterile eppendorf tubes. To prevent possible cross-contamination between samples during the PCR procedure, each microtome was cleaned with 70% ethanol before cutting the blocks, and each block was cut using a new disposable microtome blade. Tissue sections were deparaffinized by using xylene and washed with ethanol. The QIAmp DNA Mini Kit (Quiagen) was used for DNA extraction. In order to analyze the amount and quality of extracted DNA, we used spectrophotometry (Nanodrop). Samples with fewer than 15 nanograms/μl were discarded, and new ones obtained using more paraffin sections.

Polymerase chain reaction (PCR) of the ß-globin human gene sequence, using a pair of primers named Hg063/064 (Saiki et al., 1985) was performed to evaluate the quality of the DNA isolation procedure. The results indicated that 28 cases had marked DNA degradation and they were excluded from further analysis. In the remaining 72 of the 100 samples, ß-globin amplification was achieved. PCR reactions were performed in a final volume of 50 μl, containing 200 ngr of extracted DNA. Two pairs of consensus primers, My09/My11 and Gp5+/Gp6+ of the L1 gene (Table 1),

were used according to conditions that have been previously described (Karlsen et al., 1996). For negative controls, PCR was carried out in the absence of the template and in the presence of DNA isolated from the HPV negative cell line Hacat. HeLa cells were used as positive controls. PCR products were analyzed by electrophoresis on 2% agarose gels and the product was visualized with ethidium bromide staining. The My09/My11 primers amplify a 450 pairs of bases (bp) fragment from the L1 region of the HPV genome and Gp5+/Gp6+ primers amplify a 150-bp fragment from the L1 region.

Samples with positive HPV DNA were typed using the method described by Sanger et al. (1977). Sequencing was performed with Kit Big Dye Terminator v3.1 cycle sequencer and the sequences were analyzed with the sequencer capillary ABI Prism 3130 Genetic Analyzer. Automated sequencing followed by Basic Local Alignment Search Tool (Blast) was performed to determine HPV genotypes (Altschul et al., 1997).

Results

Enough well preserved DNA amplifying the hemoglobin gene Hg 064/063 was obtained from 72 of the 100 malignant or suspected of malignancy penile lesions selected. From the 72 samples obtained, 64 corresponded to squamous cell carcinoma. A study of the pathology reports from the 64 samples demonstrated that some samples corresponded to the same patient: 10 patients had 2 samples, 1 patient had 3 samples, and 1 patient had 4 samples. Thus, the total number of patients studied with penile carcinoma was 49.

The 49 cases of malignant penile tumors were further classified histologically using the classification described by Cubilla (Cubilla et al., 2001). We found that 24 cases (48.9%) were usual type squamous cell carcinomas (Fig. 1B), 1 case (2%) was a basaloid carcinoma (Fig. 1C), 1 case (2%) was a sarcomatoid carcinoma (Fig. 1D), 18 cases (36.7%) were warty carcinomas (Fig. 2C and 3), 4 cases (8,1%) were papillary carcinomas (Fig. 2B), and 1 case (2%) was a verrucous carcinoma (Fig. 2A)

PCR analysis using primers My09/My11 and

Table 1. Sequence of primers used for DNA amplification of the viral DNA.

PRIMER	SEQUENCE	AMPLIFY FRAGMENTS (bp)
My 09	5´-CGT CCM ARR GGA WAC TGA TC-3´ (*)	450
MY 11	5´-GCM CAG GGW CAT AAY AAT GG-3´ (*)	450
Gp5+	5´-TTT GTT ACT GTG GTA GAT ACT AC-3´	150
Gp6+	5´-GAA AAA TAA ACT GTA AAT CAT ATT C-3´	150

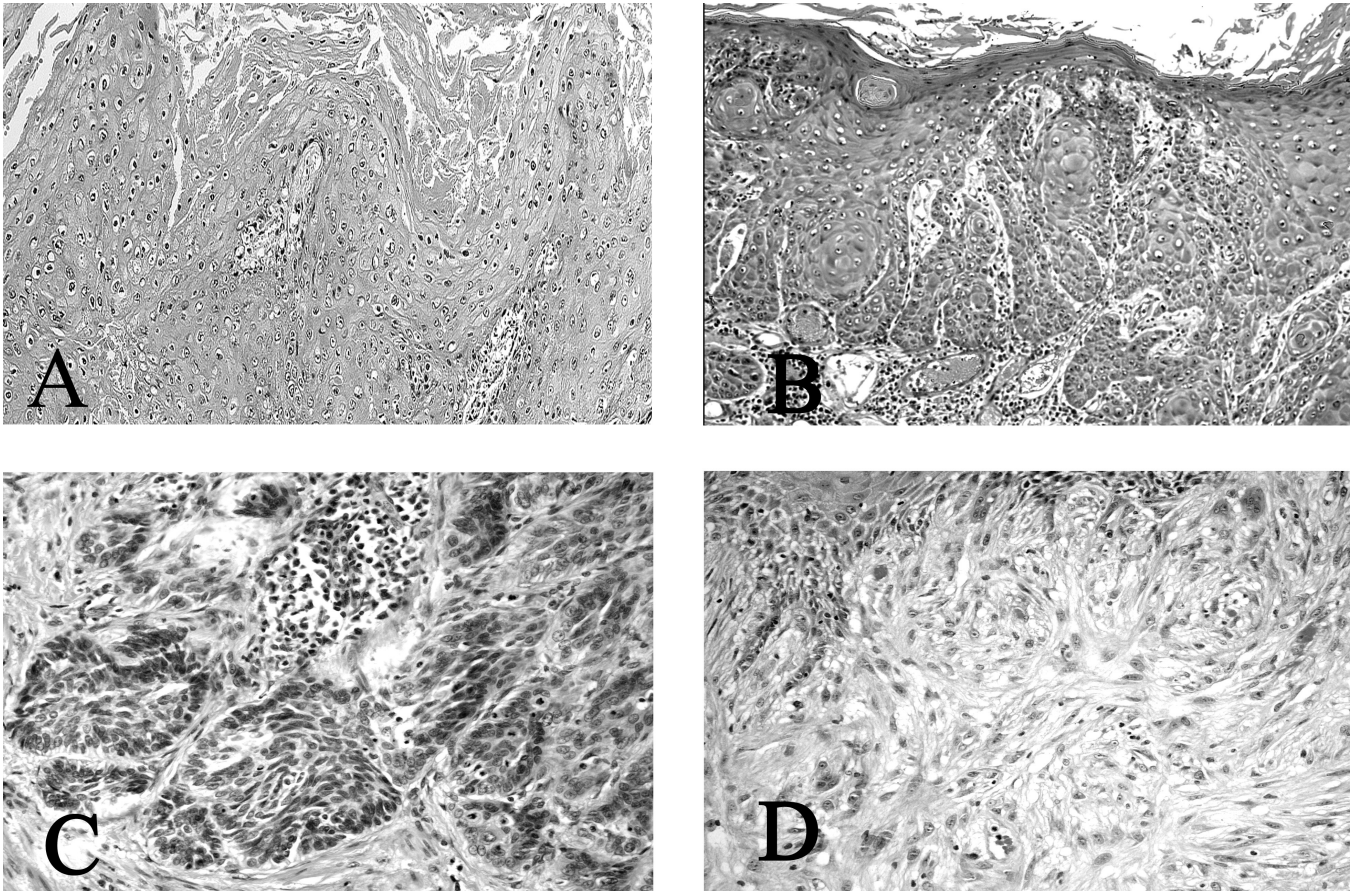(*) Variations in the sequence of Primers My 09/11 are: M=A,C; R=A,G; W=A,T; Y=C,T.

Fig. 1. Histological types of penile carcinoma. **A.** Verruciform carcinoma. x 100. **B.** Usual type squamous cell carcinoma. x 100. **C.** Basaloid carcinoma. x 200 **D.** Carcinosarcoma. x 200
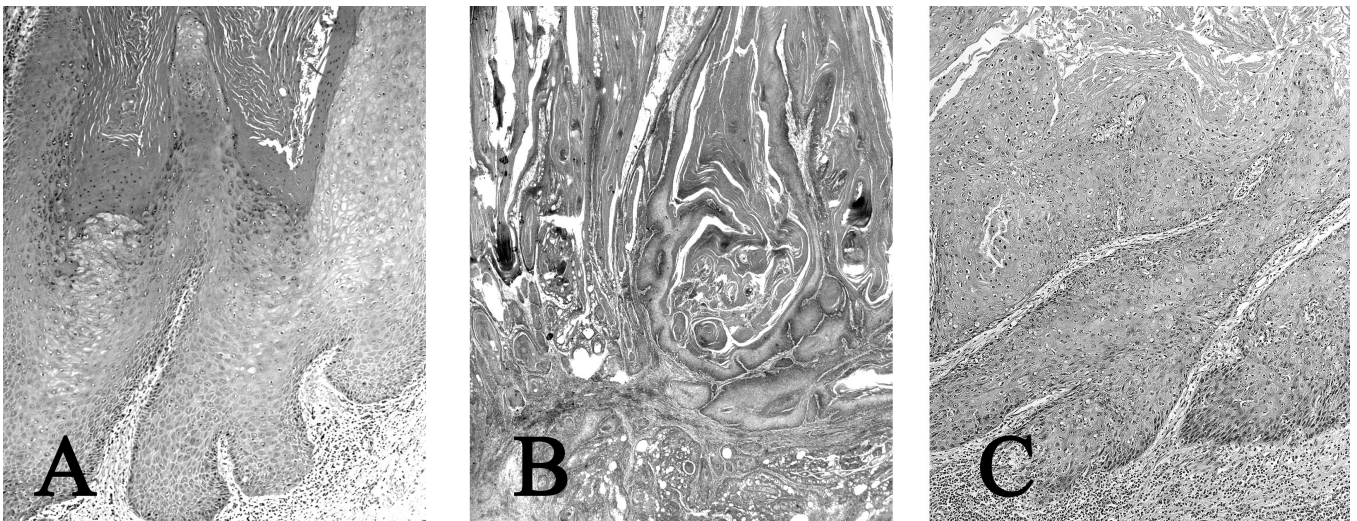

Fig. 2. Histological subtypes of penile verruciform carcinomas. **A.** Verrucous carcinoma. x 100. **B.** Papillary carcinoma. x 40 **C.** Warty carcinoma. x 100

GP5+/GP6+ was performed on the total number of samples selected with well preserved DNA. We found that 17 of the 64 samples were positive for both sets of primers, 22 of the 64 samples were positive only for primer My09/My11 (Fig. 4), and 7 samples were positive only for primer GP5+/GP6+ (Fig. 5). The total number of lesions that presented with HPV was 71,9% (Table 2).

Once the samples were assigned to each patient, we found that 38 of the 49 patients were positive for HPV (77.5 %), and 11 were negative (22.5 %). Of the HPV positive patients, genotype HPV16 was detected in 32 cases, HPV18 in 4, and in 2 subjects, results were inconclusive (Table 3). Besides HPV16 or 18, there were no other genotypes detected in any of the cases of penile carcinoma; however, HPV6 and HPV11 were found in benign penile lesions that were run in parallel. In the cases with more than one sample, the detected HPV genotypes were always concordant.

The results of HPV detection in the different histological subtypes of penile carcinomas were as follows: 17 of the 24 cases of usual type penile
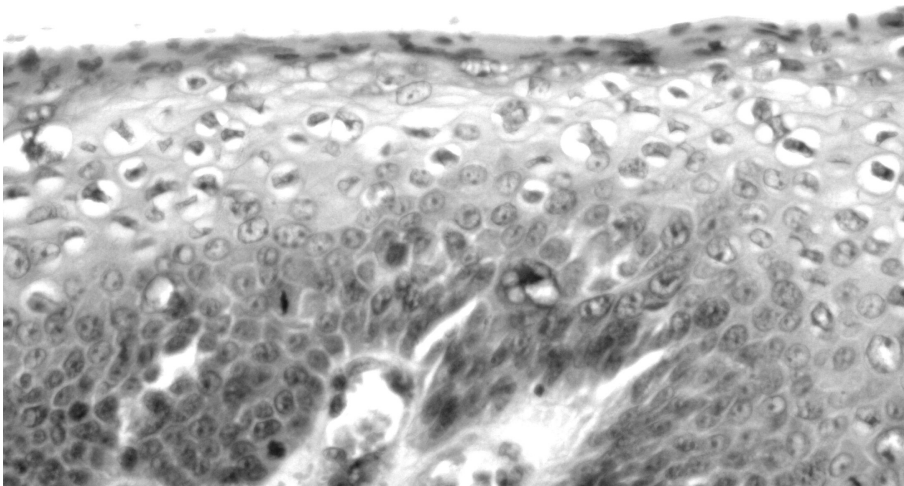

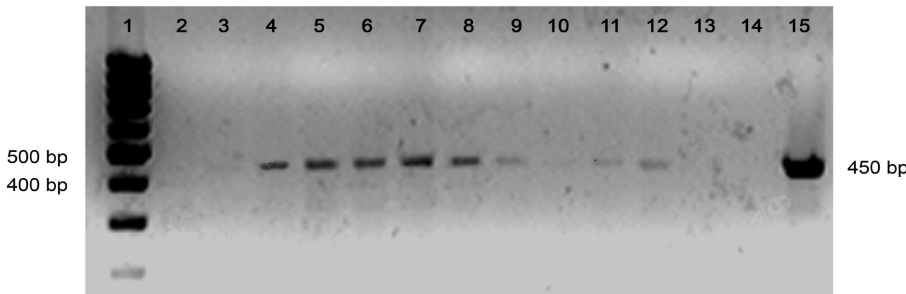**Fig. 3.** Coilocytic changes present in a case of warty carcinoma. x 200

Fig. 4. Agarosa gel of some cases amplified using primers My09/My11. Lane 1: Molecular weight marker, SIGMA 100 bp; lane 2: H_2O negative control; lane 14: CHacat negative control; lane 15: CHela Positive control; Lanes 3 to 13: clinical samples. Amplification product 450 bp.

Fig. 5. Agarosa gel of some cases amplified using primers GP5+/GP6+. Lane 1: Molecular weight marker, SIGMA 100 bp; lane 2: H_2O negative control; lane 14: CHacat negative control; lane 15: CHela Positive control; Lanes 3 to 13: clinical samples. Amplification product 150 bp.
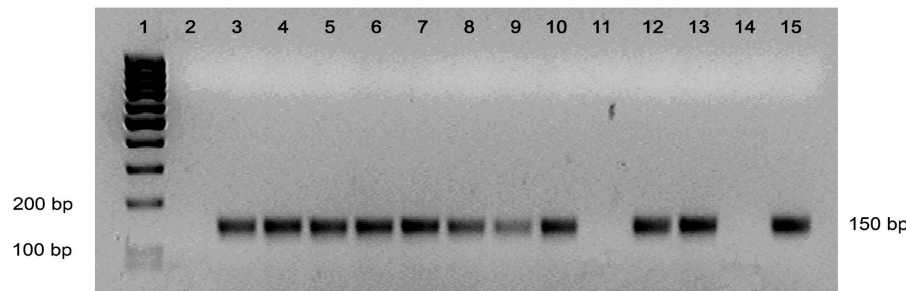
Table 2. Number and percentage of positive cases using different sets of primers.

	POSITIVE CASES
ONE or BOTH PRIMERS +	46/64 (71.9%)
ONLY MY 09/11	39/64 (60.9%)
ONLY Gp5+/Gp6+	24/64 (37,5%)

Table 3. HPV found in the 49 cases of penile carcinoma, and HPV genotypes found in the 38 positive cases.

	CASES	PERCENTAGE
HPV +	38/49	77.5%
HPV 16	32/38	84.2%
HPV 18	4/38	10.5%
Inconclusive.	2/38	5.3%

carcinomas showed HPV positivity (70.8%) (13 HPV 16, 3 HPV 18 and 1 inconclusive), 16 of 18 cases of warty carcinomas showed HPV (88.8 %) (14 HPV 16, 1 HPV 18, and 1 inconclusive), 3 of 4 cases of papillary carcinomas showed HPV (75%) (3 HPV16), the 1 verrucous carcinoma did not show HPV, the basaloid carcinoma showed HPV (HPV16), and the sarcomatoid carcinoma also showed HPV (HPV16).

Discussion

It has been demonstrated that about two thirds of the persons who maintain a sexual relationship with an HPV infected partner develop the infection. The great majority of HPV infections are subclinical and may be completely healed by the immune system within one year. However, lesions that cannot be eliminated by the immune system may persist, even for decades. The persistence of HPV-induced lesions may be responsible for the development of squamous cell carcinomas (Longworth and Laimins, 2004).

The prevalence of high risk HPV in husbands of women with cervical carcinomas has been reported to be five times more frequent than in spouses of women without cervical carcinoma (Dillner et al., 2000). However, the incidence of HPV DNA in penile carcinomas that has been reported in the medical literature is lower than that in cervical carcinomas, and similar to that in vulvar carcinomas (Rubin et al., 2001). The reported incidence of HPV in penile carcinoma is highly variable, and depends on the methodology used in the study, the types of tumors selected for study, and the target population of the study. In the present investigation we found a high incidence of HPV in penile carcinomas, which may be explained by several factors. Firstly, the methodology we employed guaranteed that enough well-preserved DNA was used in

every PCR amplification. Indeed, a constant quantity of 200 ngs of DNA measured by spectrophotometry (Nanodrop), and well-preserved DNA yielding a positive reaction with the hemoglobin gene, were requirements of our study. Another reason that may explain the high incidence of HPV in the tumors we studied, is that two pairs of consensus primers were employed. Previous studies investigating the incidence of HPV in penile carcinoma with PCR, were performed using only one set of consensus primers (Villa and Lopes, 1986; Sarkar et al., 1992; Chan et al., 1994; Bezerra et al., 2001; Picconi et al., 2000; Rubin et al., 2001). However, the most commonly employed consensus primers for detecting genital HPV, My09/My11 and Gp5+/Gp6+, have shown discrepancies between them in the number of positive cases that amplify HPV DNA in clinical samples. This suggests that each set of primers has a different potential for amplifying the DNA of specific types of HPV. For this reason, the prevalence of HPV DNA in a group of clinical samples may be underestimated if only one set of consensus primers is used (Qu et al., 1997). In the present study of 64 samples using two sets of consensus primers, we found that the percentage of HPV positive cases was 71.9% (77.5% of patients). Had only one set of primers been used, the percentage of positive cases would have been 60.9% for primers My09/11, and 37.5% for primers Gp5+/6GP+. Therefore, our results indicate, for the first time, that it is advisable to use both sets of primers.

The third factor that we consider in explaining the high incidence of HPV DNA in the present study, is the presence of a high proportion of warty type penile carcinomas found in our population. This type of carcinoma shows marked condilomatous changes and is strongly associated with HPV (Ouban et al., 2003).

It is generally accepted that in formalin-fixed paraffin-embedded tissue, the percentage of positive cases obtained is higher using smaller amplification products (Qu et al., 1997). Our study failed to follow this rule since we obtained a higher percentage of positive cases with My09/My11 primers generating an amplification product of 450 pb, than with Gp5+/6+ primers, generating a smaller amplification product (150 pb). A possible explanation for this finding is that GP5+/GP6+ primers amplify very low levels of HPV DNA when there is a limited number of mismatches between the primer and the sequence of the targeted HPV DNA, but when the number of mismatches is 4 or more, the sensitivity decreases one thousand times (de Roda Husman et al., 1995). When the viral genome is integrated into the genome of the host cell, an event that is usually observed in invasive cancer and in high grade lesions, many mutations and deletions of the viral DNA are produced (Van den Brule et al., 1990; de Roda Husman et al., 1995). Decreased sensitivity of the Gp5+/6+ set of primers in the detection of HPV DNA of penile carcinomas may be expected when these deletions or mutations are produced at the annealing area of the DNA (Karlsen et al., 1996; Qu et al., 1997). Such results

have not been reported using primers My09/My11, since they are degenerated consensus primers with 8 to 16 variant primers for each strand (Table 1) that may detect a wide range of HPV DNA types (Manos et al., 1989).

Our study is in accordance with most investigations reporting HPV16 as the most frequently detected genotype in penile carcinoma (Sarkar et al., 1992; Chan et al., 1994; Bezerra et al., 2001; Rubin et al, 2001). However, in some regions of South America, the most prevalent genotype appears to be HPV18 (Villa and Lopes, 1986; Picconi et al., 2000). This finding may indicate the existence of geographical/ epidemiological variability of HPV genotypes.

Sequencing of the products of amplification provides the most conclusive results for HPV genotyping. In fact, this method is considered to be the gold standard technique for determining HPV genotypes. Although it is a laborious technique, we performed sequencing in our study for its reliability. However, sequencing has a limitation, namely the identification of genotypes when two or more types of HPV are present. When a certain genotype predominates, this is the one that is typed, while other genotypes remain unidentified. In a sample without a clear predominance of any one genotype, the result may be ambiguous (Nelson et al., 2000), possibly explaining why in two of our cases, we did not obtain conclusive results.

In summary, we conclude that there is a high incidence of HPV (77.5%) in penile carcinoma, at least in the target population of this study, and that HPV 16 was the genotype most often present. Although primers MY09/11 were more sensitive than primers GP5+/6+, our results provide evidence indicating that the association of these two sets of primers notably increases the probability of detecting HPV DNA.

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