

The Environmental Cofactors in Carcinogenesis in High Risk HPV/HIV-Positive Women

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The objective of the present study was to assess the presence of human papilloma virus (HPV) in HIV-infected women, with comparison between the Papanicolaou cytologic technique and the molecular PCR technique, as well as to determine the type of HPV, to measure cellular immunocompetence and to identify the presence of risk factors for the acquisition of HPV infection. Thirty HIV-infected women were selected. Vaginal and endocervical samples were collected from 27 of them. The smears were examined by 3 experienced cytologists to diagnose the presence of HPV by the Papanicolaou technique and the results were compared to HPV detection and typing by PCR. HPV-infected patients were interviewed in order to identify the presence of risk factors for the acquisition of the virus. Eight of the 27 patients analyzed (29%) presented HPV in endocervical samples submitted to PCR, 6 of them (75%) presented HPV involving a high risk of development of cervical cancer. For 5 of these patients, the cytologic diagnosis was not confirmed by PCR. When cellular immunocompetence was related to HPV infection, PCR revealed a diagnosis of HPV in 37.50% of the patients at intermediate risk for HPV infection and in 83.33% of the patients at high risk for HPV infection. These immunologically compromised HPV-infected patients are at higher risk of developing cervical neoplasia. We showed here that PCR is adequate for HPV detection and that, if only the Papanicolaou method is used for the follow-up of these patients, we will not provide good prevention of cervical cancer.

Key-Words: HPV, HIV-infected women, cervical cancer, carcinogenesis, antiretroviral therapy.

Human papillomaviruses (HPV) are epitheliotropic and have a high affinity for the genital tract. Infection with some HPV genotypes is considered to be of high risk for the development of cervical cancer. Persistent infection with oncogenic HPV is a necessary, but not sufficient, condition for the development of cervical cancer. The joint action of other environmental cofactors with HPV is necessary to influence the transition from cervical HPV infection to cervical cancer. Among such cofactors are HIV, smoking and sexual activity [1].

Invasive cervical carcinoma in adolescents and adults, when associated with HIV-positive serology, defines the diagnosis of AIDS, indicating impaired cell immunity [2,3]. When associated with HIV infection, cervical carcinomas have proved to be more aggressive and refractory to traditional treatment, with high recurrence rates (39% in seropositive patients *versus* 9% in patients with negative serology) and with severity directly related to the degree of immunosuppression. They also present an uncommon pattern of metastasis and are related to a poorer prognosis than those not associated with HIV [4,5].

The study of HPV infection among HIV-infected women has attracted the attention of investigators because of the prevalence about 5 times higher of this infection among them

compared to non-HIV-infected women with a high risk behavior. This interest is focused on the high potential of HPV infection to associate with cervical neoplasias and their precursor lesions among HIV-infected women, at different frequencies [5,6]. HIV infection probably acts as one of these factors, activating cell transformation by still poorly understood mechanisms or by depressing local immunity in the genital tract [1].

Peripheral blood CD₄⁺ lymphocyte counts have prognostic implications in the evolution of HIV infection since they represent a measure of cellular immunocompetence. In the United States, the Centers for Disease Control and Prevention emphasizes the importance of CD₄⁺ lymphocyte counts in the characterization of the clinical conditions related to HIV, with counts of less than 200 cells/mL blood or a percentage of less than 14% of total lymphocytes being considered critical. In Brazil, the Ministry of Health has established that lymphocyte counts of 50 to 200 cells/mm³ characterize a stage with a high probability of the onset of opportunistic diseases [3].

Considered as a whole, data reported by Palefski et al. [6] suggest that measurements of immunity (CD₄⁺ lymphocyte counts and HIV viral load) together with HPV typing, particularly when HPV is detected persistently, could be valuable for the identification of HIV-infected women at high risk of developing cervical neoplasia. They should be followed up by frequent cytologic examinations and submitted to more sensitive methods for the detection of HPV infection.

Therapeutic agents for the treatment of HIV infection are currently available on the market. The use of these medications has become known as highly active antiretroviral therapy (HAART). One of the main objectives of HAART is to reduce the viral load to undetectable levels (below 50 to 80 copies/mL) within a period of 6 months, delaying the progression of

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immunodeficiency and/or restoring immunity as much as possible, increasing the time and quality of life of the infected person. The efficiency of HAART can be evaluated on the basis of its capacity to reduce the viral load to satisfactory levels, i.e., below the detection limit, after 6 months. A 99% reduction of the initial viral load after 12 to 16 weeks is considered to be a positive result of HAART [3].

The patients studied in the present investigation are at high risk of developing cervical cancer because of their important risk factor represented by AIDS. The objective of the present study was to determine the presence of HPV in these women, with a comparison of a cytologic technique (Papanicolaou) and a molecular technique (PCR), as well as to determine the type of HPV and to measure cellular immunocompetence and other associated risk factors such as smoking, sexual activity, type of delivery, and use of contraceptive methods.

Materials and Methods

Thirty HIV-infected women attended at the Service of Infectious-Contagious Diseases in Gynecology and Obstetrics (SEMIGO) and at the Special Unit of Infectious Diseases (UETDI), both belonging to the University Hospital, Faculty of Medicine of Ribeirão Preto (HC-FMRUSP), were selected. The patients were submitted to gynecologic examination, including the collection of vaginal and endocervical samples (Papanicolaou) and an endocervical smear for the analysis of HPV DNA [9].

For the diagnosis based on the Papanicolaou method, 3 cytologists examined the smears and the results obtained were compared to the detection and typing of HPV by PCR according to the protocol of Gravitt et al. [9].

As an internal control of PCR, a 268 bp fragment of the b-globin gene was amplified using the GH20/PC04 primers in order to assure DNA integrity. As a negative control, the samples were replaced with water [10,11]. One of the initial samples, denominated 6T0, was tested in the Laboratory of Virology of the Ludwig Institute for Cancer Research. The positive control used was the DNA of a human cell line (HeLa) with the HPV genome integrated serotype 18. This sample was used as the control of amplification of both the fragment relative to HPV and the fragment relative to the b-globin gene. The samples were submitted to 2% agarose gel electrophoresis for 50 minutes, with constant 100 mA amperage, followed by staining with 1% ethidium bromide. The samples were compared to the positive control cited above and those showing amplification of the 450 bp segment of HPV and of the 268 bp segment of b-globin were considered to be positive. The samples for PCR were typed by restriction fragment length polymorphism (RFLP) produced by the restriction enzymes Bam H I, Hae III, Hin f I, Pst I, RSA I (Amersham Pharmacia Biotech, Little Chalfont – Buckinghamshire, UK) and Dde (Roche, F.Hoffmann – La Roche LTD Corporate Communications Grenzacherstrasse - Switzerland), according to the method of Bernard et al. [12]. The products of enzymatic

digestion were separated by 10% acrylamide gel electrophoresis for 90 minutes at constant 100 mA amperage. After ethidium bromide staining and visualization under UV light, the patterns were compared to the DNA molecular weight standard, FX174 RF DNA/Hae III Fragments (Invitrogen Life Technologies, Carlsbad, California). The viral types were compared to the standard described by Bernard et al. [12].

Epidemiological and socioeconomic data were obtained by a standard interview using a structured questionnaire. The patients were asked about their sexual behavior (number of partners along life, and number of partners during the last year and during the last 6 months), the type of contraceptive method used, the type of delivery, smoking habit, and the consumption of alcoholic beverages. Patients younger than 18 years and virgin patients were excluded from the study, which was approved by the Ethics Committee on Research of the Clinical Hospital of the School of Medicine of Ribeirão Preto, University of São Paulo. All subjects were volunteers and gave written informed consent.

Results

PCR and Viral Typing

Thirty HIV-infected patients were selected for the present study. Three of them did not attend the medical visits and were excluded from the study. Twenty-seven women were submitted to gynecologic examination and to collection of an endocervical smear. The material was submitted to cytological exams (triple cytology or Papanicolaou test) and to PCR for the diagnosis of HPV.

Among the samples tested by PCR, 8 were positive for HPV DNA (patients 1, 2, 3, 6, 9, 10, 15, and 16). Amplification of fragments of approximately 450 bp, characteristic of HPV, occurred in 29.6% of the cases (8/27), as shown in Figure 1.

For cytological examination, each sample was tested by 3 different cytologists who were unaware of the serologic status of the patients regarding HIV. The first examiner detected a picture suggestive of HPV in 8 patients (numbers 2, 10, 11, 13, 15, 18, 23 and 24), the second detected it in 4 patients (numbers 2, 11, 13 and 23) and the third detected it in only 1 patient (number 13). These results are presented in Table 1.

Of the 8 patients found to be positive for HPV DNA by PCR (Figure 1), only patient number 10 received a diagnosis of class III, suggesting moderate dysplasia. Patient number 13 showed moderate dysplasia according to the Papanicolaou classification, but did not show the presence of HPV by PCR (Table 1).

According to the RFLP technique, the viral types detected in the 8 positive samples for HPV DNA (Figure 1) were 16, 26, 56, 59 and 69. HPV type 69 was detected in patients 1 and 9, HPV type 56 was detected in patients 2 and 16, HPV type 16 was detected in patient 10, and HPV type 59 was detected in patient 6. All of these types are considered to be of high risk for the development of cervical carcinoma [13]. These results are presented in Table 2. HPV type 26 was detected in patients 3 and 15; this type is considered to be of low risk for the

development of cervical carcinoma, being more associated with benign lesions such as common genital warts [14].

Epidemiologic Characteristics

The epidemiological data of the patients are presented in Table 3. Patient age ranged from 18 to 52 years, mean age was 33 years and the most frequent age range was 18 to 39 years (66%-18/27). Married and cohabiting women corresponded to 51.8% (14/27) of the participants in the study.

Mean age at first sexual relation, with vaginal penetration, was 17 years (range: 12-33). A considerable proportion of these women (88%, 24/27) had their first sexual experience between 12 and 22 years. Six participants (22.2%, 6/27), who had been or still were sex professionals, were unable to establish the number of sex partners with whom they had had contact. For 17 participants, the mean number of sex partners was 3 along life. The mean number of partners during the last 6 months of the last year was less than one (0.9), since 4 participants did not have sexual relations during the study period and 1 of them reported that she still was a sex professional.

Thirteen participants (13/27-48%) were taking or had been taking oral contraceptives (OC) for a mean time of 48 months (range: 7 months to 10 years). Regular use of a preservative by their partners was reported by 10 patients (48%-13/27), and the concomitant use of a preservative and an OC was reported by 3 of them. Two participants had submitted to tube ligation as a contraceptive method.

Eight women (29.6%-8/27) reported having had a sexually transmissible disease (STD) and 8 (29.6%-8/27) reported that their partner had suffered a STD.

Sixteen patients (59.2%-16/27) declared that they were or had been smokers, most of them (51.8%-14/27) consuming 10 to 30 cigarettes a day. Smoking started, on average, at 15 years of age and was maintained for a mean time of 16 years (range: 5 to 31 years).

The mean number of pregnancies and of liveborn children was 3. Four patients had at least one abortion. Vaginal delivery was the predominant type, with 59.2% (16/27) of the participants having delivered at least 1 child by this route. Four patients were nulliparae.

Highly Active Antiretroviral Therapy (HAART)

Sixteen patients (numbers 1, 3, 8, 11, 12, 13, 15, 17, 19, 20, 21, 22, 23, 24, 25 and 26) did not receive HAART but had been followed up for a period of time ranging from 98 to 309 days. Eleven patients had received HAART during a period of follow-up ranging from 125 to 351 days. Normally, the patients had started therapy at intervals ranging from 7 to 351 days after the initial visit (numbers 2, 4, 5, 6, 7, 9, 10, 14, 16, 18, and 27) Five of these patients (numbers 2, 6, 9, 10 and 16) were found to have HPV DNA by the PCR technique.

As shown in Table 4, all patients receiving HAART presented increased CD₄⁺ counts and decreased HIV viral loads. The Ministry of Health recommends that all patients in the early phase of treatment, as was the case for the present

patients, receive potent or triple antiretroviral schemes [3]. As shown in Table 4, the initial HAART selected for the present patients was the combination of 2 nucleoside analogue reverse transcriptase inhibitors (NRTI), lamivudine (3TC) plus stavudine (d4T) or zidovudine (AZT), and a non-nucleoside analogue reverse transcriptase inhibitor (NNRTI), nevirapine or efavirenz. Two patients (numbers 6 and 14) received a combination of AZT and 3TC (NRTI) with nelfinavir.

Discussion

Virus Detection and Typing

HPV was detected by PCR in endocervical samples from 8 of the 27 patients studied, with 6 of them (75%) presenting serotypes 16, 56, 59 and 69, of high risk for the development of cervical cancer. The HPV 16 genotype was detected in only 1 patient (number 10), the HPV 56 genotype was diagnosed in 2 patients (numbers 2 and 16), HPV 59 was detected only in patient no. 6, HPV 56 was diagnosed in 2 patients (numbers 2 and 16), and HPV 69 in patients nos. 1 and 9. Two other patients (numbers 13 and 15) presented HPV type 26, of low risk for the development of cervical cancer, corresponding to a percentage of 25%.

Among the 27 samples analyzed by the 3 examining cytologists, examiner no. 1 detected cytologic changes suggestive of HPV in patients 2, 10, 11, 13, 15, 18, 23, and 24. In 5 of these patients (numbers 11, 13, 18, 23 and 24) the cytologic diagnosis was not confirmed by PCR. Cytologist no. 2 detected cytologic changes suggestive of HPV in 4 patients (numbers 2, 11, 13 and 23). Only one of these results, for patient number 2, was confirmed by PCR. Cytologist number 3 detected cytologic changes suggestive of HPV only in patient 13 and this result was not confirmed by PCR. This was due to the fact that cytopathologic examination, which has been widely used and accepted as a screening method for the prevention of cancer of the cervix, has a low sensitivity of about 70% owing to the intra- and interobserver variability involved in the exam, which leads to a large number of false-negative results [18-20]. In the present study, even though the patient sample was small, the cytopathologic examination showed only 38% sensitivity compared to PCR.

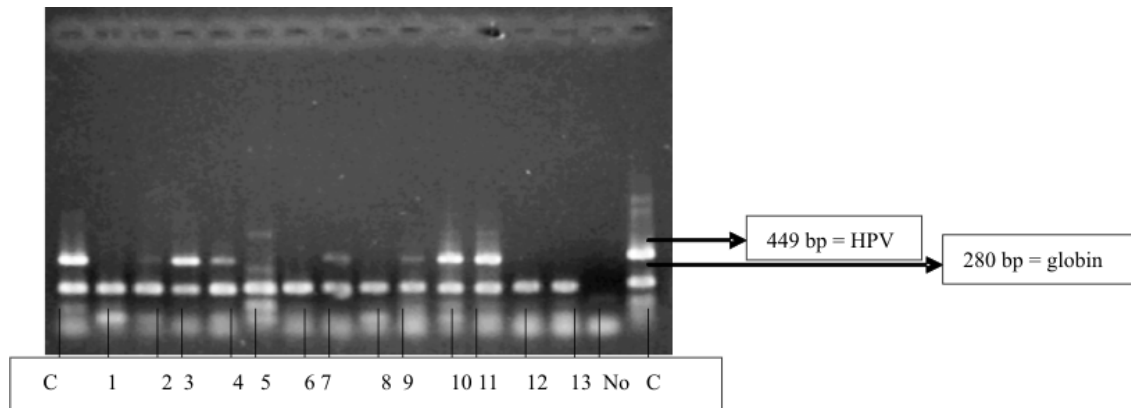
In 4 cases (patients 1, 6, 9 and 16), high risk HPV infection was detected by PCR, whereas none of the examining cytologists had detected cytologic changes suggestive of HPV infection (Tables 1 and 2).

Risk Cofactors for the Progression of HPV Infection to Cervical Cancer

Sexually Transmissible Diseases

STDs are considered to be important cofactors for the progression of HPV infection to more severe lesions [15]. All the women studied here, by being infected with HIV, were already under the influence of this cofactor. Eight patients (8/27-29.6%) reported having been infected with some STD other than HIV infection and 8 (29.6%) reported that their partners had been infected with an STD other than HIV infection (Table

Figure 1. Product of the amplification of the viral genome of HPV generated after PCR.



C=positive control; the numbers from 1 to 13 refer to patients 1 to 13; No=negative control; 268 bp=amplification of the human β -globin gene used as internal control; 449 bp=amplification of the HPV gene. All samples were tested in duplicate on different dates in a random manner.

Table 1. Comparison of the results obtained by the molecular (PCR) and cytologic (Papanicolaou) techniques

Patient	C1		C2		C3		PCR
	HPV	Class	HPV	Class	HPV	Class	
1		II				I	positive
2	positive	II	positive			II	positive
3		II		II		II	positive
4		II		II		II	
5		II		II		II	
6		II		I		I	positive
7		II		II		II	
8		II		II		II	
9		II		II		II	positive
10	positive	III		II		II	positive
11	positive	II	positive	II		II	
12		II		II		II	
13	positive	III	positive	II	positive	II	
14		II		II		II	
15	positive	II		II		II	positive
16	**						positive
17		II		I		I	
18	positive	II		II		II	
19		I		I		I	
20		I		I		I	
21		II		II		II	
22		I		I		I	
23	positive	II	positive	II		II	
24	positive			II		II	
25		I		II		II	
26		II		I		II	
27		**					

C1=examining cytologist 1; C2=examining cytologist 2; C3=examining cytologist 3. The patients whose results are blank presented other cytologic results not associated with the molecular or cytologic diagnosis of HPV. **The material obtained from patients 16 and 27 for cytologic examination was unsatisfactory for the above exams.

Table 2. Types of human papillomavirus detected by the restriction fragment length polymorphism technique in the samples from HIV-infected patients and their respective risks for the development of cervical cancer

Patient	HPV Type	Risk
1	69	high
2	56	high
3	26	low
6	59	high
9	69	high
10	16	high
15	26	low
16	56	High

Table 3. Epidemiologic and socioeconomic (socioeconomic behavioral and reproductive history)

Characteristic	N/Total	%
Age (years)		
18-25	4/27	14.8
25-32	10/27	37.0
32-39	4/27	14.8
39-46	5/27	18.5
≥46	4/27	14.8
Marital status		
Cohabiting	10/27	37.0
Married	4/27	14.8
Widow	3/27	11.1
Single	6/27	22.2
Separated/Divorced	4/27	14.8
Age at first sexual relation		
12-17	14/27	51.8
17-22	10/27	37.0
22-27	2/27	7.4
≥32	1/27	3.7
Number of partners/life		
1-3	13/27	48.1
3-5	3/27	11.1
5-7	2/27	7.4
7-8	3/27	11.1
Undefined	6/27	22.2
Sexually transmissible diseases (STD)		
Patients		
No/Unknown	16/27	59.2
Lesions associated with HPV	3/27	11.1
Syphilis/herpes	3/27	11.1
Unspecified STD	5/27	18.5
Partners		
No/Unknown	19/27	70.3
Lesions associated with HPV	4/27	14.8
Unspecified STD	4/27	14.8

Table 3. (Continued)

N of cigarettes/day	0	
< 10	11/27	40.7
10-20	3/27	11.1
20-30	7/27	25.9
20-30	4/27	14.8
≥40	2/27	7.4
Type of delivery*		
Cesarean section	5/23	21.7
Normal	8/23	34.7
Normal and cesarean section	8/23	34.7
Forceps	1/23	4.3
Normal and forceps	1/23	4.3

*Four nulliparous patients.

3). However, this information is of relative value since it was obtained by interviews with the patients. This type of information is influenced by multiple factors such as the inability to understand the technical terms used in previous diagnoses, embarrassment during the interview, forgetfulness, confusion, and difficulty in dialogue and in the patient's relationship with their partners, among many others.

Vaginal Deliveries

The risk of HPV infection progressing to cancer of the uterine cervix among women younger than 45 years is directly proportional to the number of pregnancies resolved by vaginal delivery, probably due to hormonal events related to the second and third trimesters of pregnancy, such as ectopy and metaplasia in the transformation zone [16].

Due to the number and type of deliveries and to age, 3 patients (numbers 14, 15 and 22) had been exposed to this risk cofactor for neoplastic progression.

Smoking

Studies published by IARC have shown that the risk of developing cancer of the cervix for HPV-infected women is about twice higher among women with a history of smoking 20 cigarettes a day for at least 6 years [14]. Four participants in the present study (numbers 6, 15, 22 and 25) fulfilled these criteria, with infection with oncogenic HPV and a smoking history ranging from 7 to 21 years. Thus, these women were exposed to a highly relevant cofactor capable of influencing in a significant manner the progression of cervical infections to high grade lesions and invasive cervical cancer.

T-CD₄ + Lymphocyte Count and Quantitation of Viral Load

The elevated prevalence of high risk HPV among HIV-infected women is believed to be related more to immunosuppression, which leads to the persistence and reactivation of latent infections that culminate in the recent acquisition of new HPV types. The immunity determination and HPV typing are important parameters for the identification

Table 4. Type of antiretroviral therapy, HPV infection, T-CD₄+ lymphocyte count and quantification of HIV viral load in the HIV-infected patients studied

Antiretroviral therapy			Case N ^o	HPV DNA	T-CD ₄ + lymphocytes		HIV viral load	
NRTI	NNRTI	PI			T0	T1	T0	T1
D4T+3TC	Efavirenz	Nelfinavir	9	69	15	68	87.000	BDL
AZT+3TC			6	59	341	587	130.000	BDL
			14	-	283	402	20.000	4.623
AZT+3TC	Nevirapine		4	-	331	675	12.000	678
			27	-	357	##	##	##
AZT+3TC	Efavirenz		7	-	88	472	##	BDL
			2	56	20	102	##	BDL
			16	56	76	136	300.000	BDL
			18	-	12	162	132.080	BDL
			5	-	##	241	##	10.042
			10	16	62	87	299.036	130

NRTI=nucleoside reverse transcriptase inhibitor; NNRTI=non-nucleoside reverse transcriptase inhibitor; PI=protease inhibitor, AZT=zidovudine; 3TC=lamivudine; d4T=stavudine; T0=initial collection; T1=final collection, BDL=below the detection limit; ##=not available; -=negative HPV DNA; T-CD₄+ lymphocytes=(cells/mm³); HIV viral load=(copies/mL).

Table 5. Patient classification regarding the risk of HPV infection according to their respective viral load (HIV) and CD₄+ count obtained from their medical records

Risk	CD ₄ + count (cells/mm ³)	HIV viral load (copies/mL)	Patients	Patient with HPV
Low	>200	<20.000	4,5,7,11,12,13,14, 17 and 24	***
Intermediate	>200	<20.000	3,6,8,15,19, 20, 22, 23	3,6,1 and 5
High	<200		1,2,9,10,16 and 21	1,2,9,10 and 16

*** Absence of HPV determined by PCR. Patients 18, 25 and 27 are not mentioned in the table because their medical records were not available.

of HIV-infected women at high risk to develop cervical neoplasia [5].

The combination of CD₄+ lymphocyte count and HIV viral load has an excellent predictive value of the risk of HPV infection among HIV-infected women. According to Palefski et al. [5], at their lowest values (CD₄+ count of less than 200 cells/mm³ and an HIV viral load higher than 20,000 copies/mL), both parameters are important for the activation and replication of HPV, as well as for its detection. HIV-infected women are at low risk when their CD₄+ counts are higher than 200 cells/mm³ and their viral loads lower than 20,000 copies/mL. Women whose CD₄+ counts are higher than 200 cells/mm³ and whose viral loads are higher than 20,000 copies/mL are at intermediate risk, and women with CD₄+ lower than 200 cells/mm³ regardless of viral load are at higher risk.

Measurement of Immunity

According to Table 4, which relates cell counts, HIV viral load and risk for HPV infection, none of the 9 patients considered to be at low risk for HPV infection presented a positive result for the diagnosis of HPV by PCR. Of the 8 patients considered to be at intermediate risk for HPV infection, 3 (numbers 3, 6 and 15) were found to be HPV positive by PCR, corresponding to 37.50% (3/8). Of the 6 patients considered to be at high risk for HPV infection, 5 (83.33%) (numbers 1, 2, 9 and 16) were found to be HPV positive by PCR.

Cytology

Walboomers et al. (1992) concluded that cytopathologic examination is of low sensitivity (about 70%), possibly leading to false-negative results. In the present study, we observed a lower percentage of agreement (38%) when we compared the 2 techniques; however, we should point out the importance of the sample size when different studies are compared. The morphologic criterion for the diagnosis of HPV infection shows low sensitivity and specificity since it is based on morphologic criteria such as the presence of hypertrophic, bi- or multi-nucleated, hyperchromatic, koilocytotic, amphophilic or dysplastic cervical cells, whose detection depends largely on the good preparation and staining of the slides and on the experience of the examiner [17]. Thus, HIV-infected patients require a more specific technique for the detection of HPV infection, rather than simply a cytologic diagnosis.

The present results show that PCR detected 8 out of 27 patients with the presence of HPV DNA, with 6 of them being infected with HPV types involving a high risk of development of cervical cancer. The cytologic technique indicated changes suggestive of HPV in only 4 of these 8 women, showing low sensitivity.

The patients who participated in the present study, by being infected with HIV and immunocompromised and by being exposed to factors such as smoking, sexual activity and

other STD, are at higher risk to develop cervical neoplasia and therefore require more sensitive techniques for the diagnosis of HPV. In the present study we showed that PCR is an adequate technique for this purpose and we also determined that the use of only the Papanicolaou test to monitor these patients will not provide good prevention of cervical cancer.

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