

Prevalence of Human Papillomavirus DNA in Female Cervical Lesions from Rio de Janeiro, Brazil

SMB Cavalcanti/⁺, ICP Frugulhetti, MRL Passos, MEF Fonseca*, LHS Oliveira

Departamento de Microbiologia e Parasitologia, Instituto Biomédico, Universidade Federal Fluminense, R. Ernani Melo 101, 24210-030 Niterói, RJ, Brasil *Departamento de Virologia, Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Ilha do Fundão, 21941-000 RJ, Brasil

A hundred-sixty paraffin-embedded specimens from female cervical lesions were examined for human papillomavirus (HPV) types 6, 11, 16 and 18 infections by non-isotopic in situ hybridization. The data were compared with histologic diagnosis. Eighty-eight (55%) biopsies contained HPV DNA sequences. In low grade cervical intraepithelial neoplasias (CIN I), HPV infection was detected in 78.7% of the cases, the benign HPV 6 was the most prevalent type. HPV DNA was detected in 58% of CIN II and CIN III cases and in 41.8% of squamous cell carcinomas (SCC). Histologically normal women presented 20% of HPV infection. Oncogenic HPV was found in 10% of these cases, what may indicate a higher risk of developing CINs and cancer. Twenty-five percent of the infected tissues contained mixed infections. HPV 16 was the most common type infecting the cervix and its prevalence raised significantly with the severity of the lesions, pointing its role in cancer pathogenesis. White women presented twice the cervical lesions of mulatto and African origin women, although HPV infection rates were nearly the same for the three groups (approximately 50%). Our results showed that HPV typing by in situ hybridization is a useful tool for distinguishing between low and high risk cervical lesions. Further studies are required to elucidate risk factors associated with HPV infection and progression to malignancy in Brazilian population.

Key words: human papillomavirus - CIN - carcinoma - *in situ* hybridization

The human papillomaviruses (HPVs) are a remarkably heterogeneous group encompassing at least 60 distinct genotypes. Among them, more than 20 types can infect the genital epithelia (de Villiers 1989). Epidemiological studies have demonstrated a close association of HPV types 6 and 11 with benign condyloma acuminata and low grade intraepithelial neoplasias (CIN I) (Gissman et al. 1983, de Villiers et al. 1987). The DNA of HPV 16 and 18 and less frequently of other types predominates in anogenital carcinomas and high grade cervical intraepithelial neoplasias (CIN III) (Pfister 1987). This biological behavior suggests that HPV 16 and 18 are of high risk for progression to malignancy while HPV 6 and 11 represent low risk types (zur Hausen 1991).

There are evidence that both benign warts and malignant anogenital cancers are increasing in prevalence in areas such as Latin America and Asia. Recent studies have also documented the widespread epidemic of HPV infections showing

prevalence rates varying from 10% to 80% depending upon the population studied and the methods used for viral detection (Copleson et al. 1987, Koutsky et al. 1988, Bejui-Thivolet et al. 1992, Moscicki 1993).

In Brazil, high annual cancer incidences of over 30 cases per 100,000 have been reported. Nevertheless, there has been a few published epidemiological studies on the prevalence of HPV DNA in female genital tract. McCance et al. (1986) while studying the northeastern states of Brazil, high risk areas for genital cancers, found HPV 16 in 49% of the penile cancers and in 40% of the cervix carcinomas. Villa and Lopes (1986) detected HPV 18 in 39% of the penile cancers examined in southern regions.

The present study focuses on the detection of low and high risk HPV types in benign, premalignant and malignant cervical lesions among the female population of the State of Rio de Janeiro, in order to determine the prevalence of certain HPV types by using *in situ* DNA hybridization.

MATERIALS AND METHODS

Specimens - The material of the present study comprises a series of 160 cases of normal, benign,

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+ Corresponding author and CNPq fellowship

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pre-malignant and malignant cervical biopsies of women diagnosed and treated at Hospital Universitário Antônio Pedro, Universidade Federal Fluminense (UFF), State of Rio de Janeiro, from 1988 to 1993. Formalin-fixed, paraffin-embedded biopsy specimens were available for studies at the files of the Department of Pathology. Five μm sections were cut onto Aminopropyl triethoxysilane (Sigma) coated slides, heated at 60°C for 2 hr and stored at room temperature (RT).

Histological diagnosis - Slides were stained with haematoxylin and eosin (HE) for histological grading of the cervical epithelial lesions into mild dysplasia (CIN I), moderate dysplasia (CIN II), severe dysplasia or carcinoma *in situ* (CIN III) and squamous cell carcinoma (SCC). The following criteria were applied: (a) loss of organization of the epithelium in three layers; (b) disorderly arrangement of the cells; (c) loss of polarity; (d) loss of differentiation; (e) disturbed nuclear/cytoplasmic ratio; (f) cellular polymorphism; (g) nuclear hyperchromatism or abnormal chromatin patterns; (h) abnormal mitosis. The diagnosis CIN 0 was made when the architecture of the epithelium was not disturbed. Thus, heterogeneous cervical lesions without apparent CIN (e.g., squamous metaplasia, inflammation) were classified under this category.

Probes - HPV 6b, 11, 16 and 18 DNAs were kindly provided by Dr de Villiers (Heidelberg). They were prepared and purified through caesium-chloride gradients. Plasmids were labeled using a nick translation kit (BRL) and biotin-11-d UTP (Sigma).

In situ hybridization - Sections were deparaffinized in two changes of xylol and two changes of 100% ethanol and washed in running tap water. Nucleic acids were unmasked by digestion with 0.5 mg/mL proteinase K (Sigma) at 37°C for 15 min. The hybridization mixture contained 5xSSC, 5% polyethyleneglycol, 0.1mg/mL denatured carrier DNA (herring sperm), biotinylated probes (10 $\mu\text{g}/\text{mL}$) and 50% deionized formamide for high stringent conditions. Each section was layered with 20 μl of hybridization mixture under a coverslip, denatured by heating at 92°C for 10 min on a heating block, and hybridized at 37°C for 2 hr. Coverslips were removed by soaking the slides in 4xSSC at RT for 10 min. The slides were further washed in 0.1xSSC / 50% formamide, 4xSSC and phosphate buffered saline pH7.2 (PBS) at RT for 10 min each.

The DNA-DNA hybrids were visualized by using streptavidin-alkaline phosphatase complex at RT for 30 min. Unbound conjugate was removed by two washes in Buffer 1 (0.1 M Tris HCl, 0.15 M NaCl, pH 7.5) for 10 min and once in Buffer 3 (0.1 M Tris HCl, 0.1 M NaCl, 50 mM MgCl₂, pH

9.5) for 5 min at RT. The slides were then incubated in NBT-BCIP dissolved in Buffer 3 at RT for 30 min in the dark. Slides were rinsed in distilled water to stop reaction, air dried and mounted in glycerine jelly without counterstaining. This method is thought to be capable of detecting approximately 50 to 100 viral genome copies per cell. Positive cells are detected by strong nuclear signal in the upper epithelial layers especially when associated with koilocytic features. Occasional intermediate and parabasal cells with or without koilocytosis also show a purple precipitate in the nuclei under light microscopy (Syrjanen 1990). HPV-positive and negative tissue biopsies were used as controls in every experiment.

Statistical analysis - The statistical significance of the results has been analyzed by using the chi-square test with the application of the Yates correction, when appropriate.

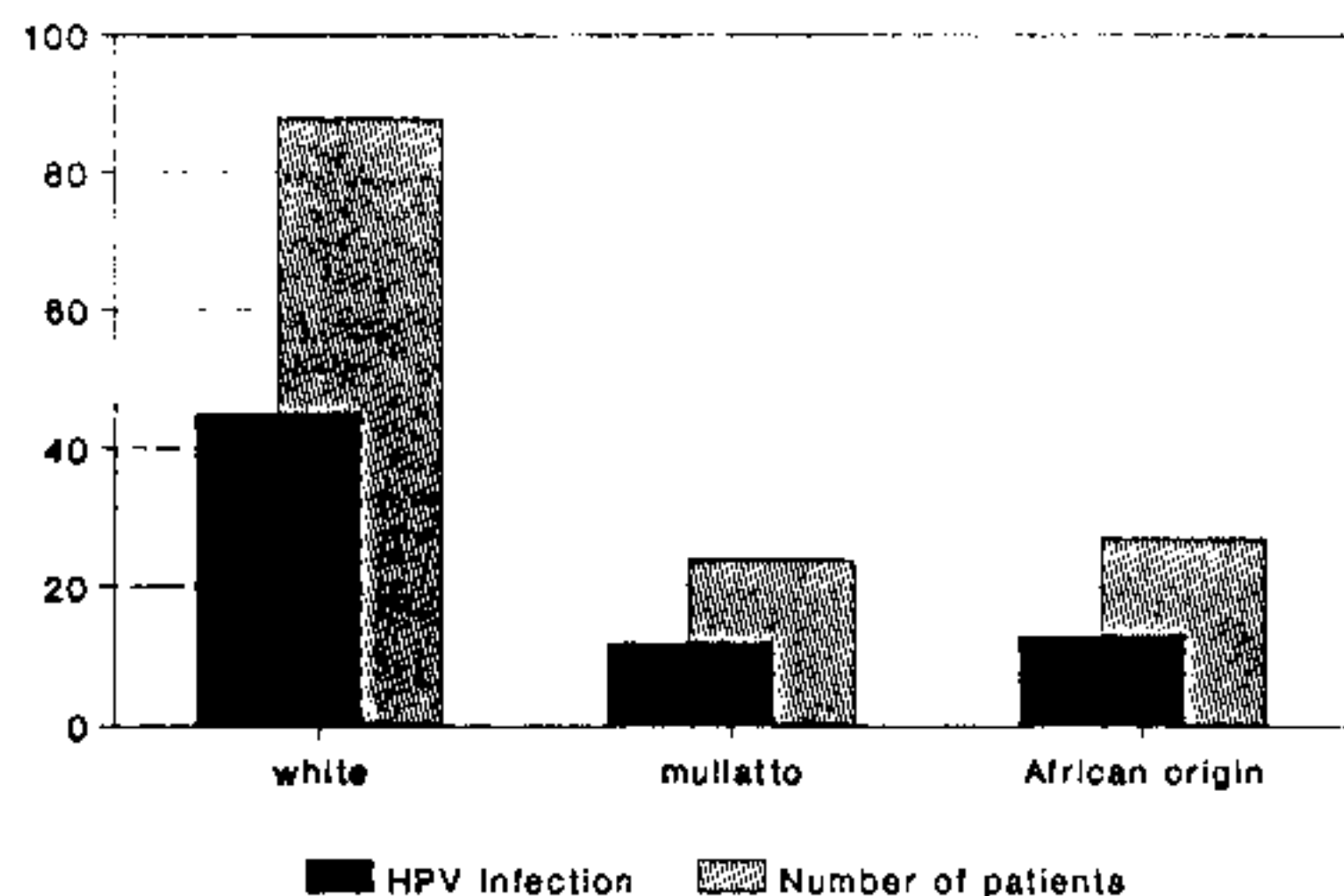
RESULTS

A hundred sixty biopsies from female cervical lesions have been investigated to detect the presence of HPV DNA. The mean age of participants was 40 years, ranging from 13 to 80 years old. The studied population was composed of 88 (55%) white, 24 (22%) mulatto and 27 (23%) African origin women.

Of the total biopsies examined, 6.3% were histologically classified as normal (CIN 0), 29.4% as low grade intraepithelial neoplasia (CIN I), 9.3% as moderate grade intraepithelial neoplasia (CIN II), 13.1% as severe neoplasia or carcinoma *in situ* (CIN III) and 41.9% as squamous cell carcinoma.

Although white patients presenting cervical lesions prevailed over African-origin and mulatto patients, prevalence of HPV infection was nearly the same for these three groups (Fig. 1). Different age distribution patterns were seen according to the histological grade of the lesions (Table I): the CIN I and CIN II ($\bar{X}_{\text{age}}=26.8$ and 34.5, respectively) groups did not exhibit statistically significant differences when compared to the control group (CIN 0, $\bar{X}_{\text{age}}=28.0$), while the CIN III and the SCC groups presented significant differences ($\bar{X}_{\text{age}}=38.9$ and 48.9; $P<0.05$ and $P<0.001$, respectively). These patterns were also seen for HPV positive lesions compared to the mean age of the groups studied (Table II).

As shown in Table III, the overall prevalence of HPV DNA in the studied group was 55% (88/160) ranging from 78.7% (37/47) CIN I to 41.8% (28/67) in SCC. In the histologically abnormal lesions (CIN I to SCC), the prevalence rate was 57.4% (86/150). The control group CIN 0 presented 20% (2/10) of HPV positivity. Statistically significant differences were detected between the



HPV infection in cervical lesions of women of different races.

TABLE I

Average age of the patients according to different histological grade of lesions

	Histological diagnosis of patients				
	CIN 0 (n=10)	CIN I (n=47)	CIN II (n=15)	CIN III (n=21)	SCC (n=67)
\bar{X} age	28.0	26.8	34.5	38.9	48.9

TABLE II

Average age of the HPV infected women according to different histological grade of lesions

	Histological diagnosis of patients				
	CIN 0 (n=2)	CIN I (n=37)	CIN II (n=9)	CIN III (n=12)	SCC (n=28)
\bar{X} age	27.0	27.5	28.4	40.3	48.1

cervical lesions and the control ($P < 0.001$) for HPV infection of the cervix.

Prevalences of different HPV types are shown in Table III. In the benign CIN I lesions, HPV 6b was the most prevalent type (35.1%) followed by HPV 16 (21.6%). In the CIN II and III, HPV 16 predominates over the others (55.6% and 50%, respectively). In the SCC, HPV 16 prevalence was the highest one (35.7%), followed by HPV 18 (32.1%) and HPV 6 (3.6%).

Among the total studied population, HPV 16 was the most common type (34.1%), followed by HPV 6b (20.5%) and HPV 18 (17%). The HPV type 11 DNA was rarely seen (3.4%). Twenty-two out of the eighty-eight (25%) HPV positive lesions showed mixed infections. They were composed by 5 HPV6,11; 4 HPV6,16; 1 HPV 6,18 and 1 HPV6,11,16,18 in CIN I; 1 HPV6,16 in CIN II; 1 HPV11,18 and 1 HPV6,16,18 in CIN III and, finally, 4 HPV6,16; 2 HPV6,18; 1 HPV11,18 and 1 HPV6,16,18 in SCC.

DISCUSSION

We have found that 88 out of 160 (55%) of all cervical specimens contained HPV DNA sequences by *in situ* hybridization. Although the use of various HPV DNA detection systems has showed variability into reported prevalence rates, our data (Table III) were consistent with those observed in other studies (Syrjanen 1989): 30 (34.1%) biopsies contained HPV 16, 18 (20.5%) contained HPV 6, 15 (17%) contained HPV 18 and 3 (3.4%), HPV 11. Assuming therefore that HPV types 16 and 18 are oncogenic, we associated the prevalence of these types with the CIN lesions. The upward trend in the oncogenic HPV prevalence rate from CIN 0 to SCC was statistically significant (Table IV,

TABLE III

HPV prevalence by histologic diagnosis

Diagnosis	Number of patients	HPV types					HPV prevalence
		6b	11	16	18	mixed	
CIN 0	10	1	-	1	-	-	2 (20.0%)
CIN I	47	13	3	8	2	11 ^a	37 (78.7%)
CIN II	15	2	-	5	1	1 ^b	9 (60.0%)
CIN III	21	1	-	6	3	2 ^c	12 (57.2%)
SCC	67	1	-	10	9	8 ^d	28 (41.8%)
Total	160	18	3	30	15	22	88 (55.0%)

^a: 5 HPV 6, 11; 4 HPV 6, 16; 1 HPV 6, 18; 1 HPV 6, 11, 16, 18

^b: HPV 6, 16

^c: 1 HPV 11, 18; 1 HPV 6, 16, 18

^d: 4 HPV 6,16; 2 HPV 6, 18; 1 HPV 11,18; 1 HPV 6, 16, 18

TABLE IV

Prevalence of oncogenic HPV types by histologic diagnosis

Diagnosis	Number of patients	HPV types		
		oncogenic ^a	others ^b	not detected
CIN 0	10	1 (10.0%)	1 (10.0%)	8 (80.0%)
CIN I	47	10 (21.3%)	27 (57.4%)	10 (21.3%)
CIN II	15	6 (40.0%)	3 (20.0%)	6 (40.0%)
CIN III	21	9 (42.9%)	3 (14.2%)	9 (42.9%)
SCC	67	19 (28.4%)	9 (13.4%)	39 (58.2%)
Total	160	45 (28.1%)	43 (26.9%)	72 (45.0%)

^a: oncogenic HPV types: HPV 16 and HPV 18^b: other HPVs: HPV 6b, HPV 11 and mixed infections

P<0,001). This increase in the prevalence of oncogenic HPVs in accordance with the severity of the cervical lesion is consistent with a role for these HPV types in the pathogenesis of cervical cancer and with the recent data described by Cornelissen et al. (1992), in which a polymerase chain reaction was used. The other HPV types appear to play a less significant role in this process, since their prevalence tended to decrease significantly from CIN I to SCC (P<0,0002). These results indicate that HPV typing by *in situ* hybridization is a useful tool for distinguishing between high and low risk cervical lesions.

In fact, *in situ* hybridization using biotinylated probes is a sensitive and appropriate method for retrospective analysis of HPV DNA sequences even in routinely paraffin-embedded biopsies (Bejui-Thivolet et al. 1992). Some researchers have focused primarily on identification of HPV DNA by using southern, dot blot and recently, the polymerase chain reaction (Gissman et al. 1983, Reid et al. 1987, Young et al. 1989). Although highly sensitive, these techniques provide no morphological information such as distribution and quantity of viral nucleic acids within lesions, because of mass tissue analysis. Syrjanen (1989) described *in situ* hybridization as being the most suitable technique for routine HPV detection, as it can be applied directly on PAP smears and punch biopsies, with the possibility of complete grading of CIN. Besides, non-isotopic probes has been proved to be equally sensitive and also preferable to isotopic ones as it is a safe and rapid method which provides an optimum resolution (Yun & Sherwood 1992).

The detection of a high percentage of multiple HPV infection (25%) is in full agreement with the finding that up to 20% of CIN lesions are infected by more than one HPV type (Crum et al.

1985, Reid et al. 1987, Syrjanen et al. 1988). The high rate of such infections may reflect a large number of sexual mates and potential exposures but it is unclear whether mixed infection is associated with a higher risk of malignancy (Evans et al. 1992).

As showed in Table III, 20% of the biopsied women with normal histology (CIN 0) were HPV positive. They may have latent infections and for the patient who had HPV 16, it might indicate a higher risk for cancer development since this HPV type is responsible for the initiation of the dysplastic transformation that leads to malignancy. Similar results have been detected by Meekin et al. (1992) and it will be interesting to investigate whether only HPV infected CIN 0 will progress to CIN lesions. Nuovo et al. (1990) stated that CIN lesions have developed in a higher proportion (71%) in patients who initially had no detectable CIN but were HPV positive.

It is also worth noting that 27% of the benign CIN I lesions showed HPV DNA types 16 or 18. The detection of potentially oncogenic HPV types in benign lesions has been described in the literature (McCance et al. 1985, Bejui-Thivolet et al. 1992). In clinical research HPV typing of such lesions is more important than in high grade CIN and SCC as highly malignant lesions are always treated by surgical excision. Thus, it is necessary to cautiously accompany these patients because they are at a higher risk of developing cancer but, in these cases it is still possible to prevent it. Nevertheless, we cannot disregard the possibility of detecting some HPV 16 and 18 variants less oncogenic than the prototypes, as recently suggested by Fang et al. (1993).

The incidence of cervical cancer varies greatly in different countries. In Brazil, high annual incidences have been reported. The association between anogenital cancers and HPV infection has been

described by several authors. Durst et al. (1983), using blot hybridization, found HPV infection rates of 45% and detected oncogenic HPV 16 in 35% (8/23) of SCC among Brazilian women. McCance et al. (1986), also using blot hybridization, reported HPV 16 in 40% (8/19) of cervical carcinomas in women from northeastern states, none of these cases presented HPV 18. On the other hand, Boshart et al. (1984) detected HPV 18 DNA in 25% of the cervical disease in Brazilian women. Even higher rates of oncogenic HPV infection should be expected by using more sensitive methodologies.

In our study, we described the presence of HPV 16 DNA in 34.1% of all cervical lesions. It is noteworthy that either cervical lesions or HPV infections showed to be age-dependent (Table I, II). This pattern could not be observed for the race since HPV infection rates were nearly the same (50%) for the three groups analyzed (Fig.). Nevertheless this is particularly interesting to notice that cervical lesions were more common in white women than in African origin or mulatto women since the most part of the population attending our hospital is composed by African origin and mulattoes. These data are in agreement with those from de Almeida (1992) who found a higher prevalence of anogenital lesions compatible to HPV infection in the white population of Rio de Janeiro.

In conclusion, we demonstrated a close association between histological grading of the lesions and specific HPV types in cervix of Brazilian women. Our results do not significantly differ from other authors with respect of geographical distribution of HPV infections, although further studies are required in order to determine specific risk factors associated to HPV infection and progression of histologically normal tissue and low grade CINs to malignancy.

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