

HPV31 and HPV33 incidence in cervical samples from women in Recife, Brazil

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ABSTRACT. Human papillomavirus (HPV) has been extensively studied concerning genomic structure, infection mechanisms, and diversity of types, as well as disease progression stages and development of vaccines. HPV type prevalence can differ in specific populations in different countries, according to ethnicity. This is the first report of an integrated project to evaluate the incidence of HPV types in different regions in Brazil in order to obtain data for vaccine development. Cervical samples were collected from women seen at a public hospital in Pernambuco, Northeast Brazil, for routine evaluation of genital alterations. Selection of the patients was random. There was a strong prevalence of HPV16 and a high incidence of HPV types 31 and 33. These

data foster the discussion about the need to evaluate viral prevalence in each geographic region in order to develop targeted vaccine programs.

Key words: Human papillomavirus; Cervical cancer; Genotyping; Prophylactic vaccines

INTRODUCTION

Human papillomavirus (HPV) has been the object of numerous investigations owing to its high mortality rates in females worldwide. The virus causes lesions in genital, upper respiratory, digestive tract, and skin tissues. If untreated, infection can develop into cancer and metastasis. More than 130 distinct HPV types have been described, and 18 are high risk due to association with genital cancer (Calleja-Macias et al., 2009).

The association between certain types of HPV and cervical cancer etiology is well established. HPV types may be classified as high, medium and low risk according to the lesion's severity (Stanley, 2001; Cubie, 2007). HPV types 6 and 11 are low risk since they only cause single or multiple genital warts. The most common high-risk HPVs are types 16, 18, 31, and 33 related to cervical, vulvar, vaginal, anal and penile cancer (zur Hausen, 2006).

HPV infections are related to the differentiation of the stratified epithelium (Pyeon et al., 2009). HPV enters the organism through local micro-lesions in basal epithelial cells and reaches the squamous cells through the virus cycle. Viral capsid disintegration occurs during the process coupled to the activation of early and late genes, and thus to the formation of virions in the apical epithelial layer (Wright et al., 2006; Szalmás and Kónya, 2009).

The viruses are mainly sexually transmitted, and certain factors, such as immunological suppression, oral contraceptives, smoking, multiple pregnancies, and vitamin deficiency, can increase chances of infection and act as cancer co-factors (Trottier and Franco, 2006; Wang et al., 2009; Al Daraji and Smith, 2009).

The development of vaccines requires better understanding of the HPV life cycle (Pyeon et al., 2009). Important studies on vaccines or virus-like particles (VLPs), produced by cloning capsid virus genes have been undertaken to increase the efficiency in HPV detection and treatment (Lowndes, 2006). VLPs are indistinguishable from native virions, preserve the conformational epitopes needed for developing neutralizing antibodies, and are able to trigger cell response (Wood et al., 2006; Hildesheim et al., 2006; Massad et al., 2009; Handisurya et al., 2009).

The quadrivalent vaccine with VLPs of L1 of types 6, 11, 16, and 18 and the bivalent vaccine with VLPs of L1 of HPV types 16 and 18 are the two commercial vaccines currently available on the market, and the costs are very high to make improvements in public health programs (zur Hausen, 2006).

HPV type 16 has been reported as the most prevalent in different Brazilian regions. The second more prevalent virus type discussed is type 18. HPV16 and HPV18 are included in two separate phylogenetic groups (Calleja-Macias et al., 2009). A previous study reported different HPV incidences in the different Brazilian regions: Lorenzato et al. (2000) discussed the major incidence of HPV31 and HPV33 in samples collected from women in Recife, Pernambuco. In this study, the aim was to determine the prevalence of high-risk HPV types in Recife, PE, Brazil. These results are highly relevant for public policies in HPV vaccination, which should take into account the virus types involved in the pathogenesis.

MATERIAL AND METHODS

Characteristics of the samples studied

Samples from females were provided by the Instituto Materno-Infantil Prof. Fernando Figueira (IMIP), from the metropolitan area of Recife, PE, Brazil, where they were selected at random. The significance of the study was explained to the patients, and written informed consent was obtained. The study was approved by the Ethics Committee of the IMIP. Cervical samples of 617 women were included in the study (median age 23 years, range = 18-35, inter-quartile range = 20-27). The patients were seen for routine evaluation due to abnormalities found in a Pap smear.

After scraping the endocervix area with a cytobrush, the samples were stored in 10 mM Tris-HCl and the DNA was extracted with the Genomic Prep Blood DNA isolation kit (GE Life Science), following the manufacturer protocol.

HPV analysis

HPV detection in the material collected was performed by polymerase chain reaction (PCR), using generic primers MY09/11, and the products were visualized by gel electrophoresis (Rambout et al., 2007). Previously, the DNA obtained from the collected samples was submitted to amplification of human β -globin sequence (primers PC04 and GH20) in order to verify the possibility of further PCR tests. Positive and negative controls were used in all procedures. The reaction mix was prepared with 5 μ L DNA, 1.6 μ M of each primer and 12.5 μ L MasterMix, in a total volume of 25 μ L. The amplification was carried out according to the following program: 94°C for 30 s, 55°C for 1 min and 72°C for 1 min for 34 cycles, followed by 10 min up to the end at 72°C. Specific PCR primers for HPVs 16, 18, 31, and 33 were also used in PCR. The reaction mix was prepared in a total volume of 20 μ L containing 4 μ L DNA, 0.4 μ M of each specific primer and 10 μ L MasterMix. Amplification consisted of 35 cycles, with the following protocol: denaturation at 95°C for 30 s, primer annealing (temperatures varied according to primers) at 57°C (for HPVs 16 and 18) and 55°C (for HPVs 31 and 33) for 30 s, and primer extension at 72°C for 1 min, followed by final extension at 72°C for 10 min. The PCR products were visualized on a 2% agarose gel, with ethidium bromide under UV light.

Some patients were selected at random for conventional cytology screening to compare with HPV detection. The samples were collected and the swab material was smeared on glass slides using the cytobrush. The material was fixed in 95% ethanol and stained following the Papanicolaou method for analysis. The types of lesions were classified by cytological analysis based on the Bethesda classification of 2001.

RESULTS

PCR analysis for HPV detection was carried out with the DNA samples that were positive for the amplification of human β -globin primers (PC04 and GH20): 509 samples were qualitatively viable for analysis. The first analysis for HPV detection was performed using generic primers MY09 and MY11. A total of 288 (56.58%) samples were verified as showing HPV sequences. In the second procedure of amplification, the DNA of the samples shown to be positive for the presence of HPV sequences were submitted to amplification using specific primers to identify HPV16, HPV18, HPV31, and HPV33 types. With the use of the specific primers, it was

possible to identify a single HPV type in 213 samples: 168 were infected with HPV16 (78.87%), 6 with HPV18 (2.82%), 33 with HPV31 (15.49%), and 6 with HPV33 (2.82%) (Table 1).

Table 1. Detection of HPV sequences in group of patients selected at random: samples with only one virus type and samples showing simultaneous infections.

Viral type	Positive samples (%)	Samples showing simultaneous infection (%)	
HPV16	168 (78.67%)	HPV16/18	2 (2.67%)
HPV18	6 (2.82%)	HPV16/31	53 (70.67%)
HPV31	33 (15.49%)	HPV16/33	14 (18.66%)
HPV33	6 (2.82%)	HPV18/31	2 (2.67%)
		HPV16/18/31	1 (1.33%)
		HPV16/31/33	3 (4%)
Total	213 (100%)		75 (100%)

Considering the 288 samples in which it was possible to identify HPV sequences, 75 samples displaying more than one HPV type were detected: the results indicated that the association of types 16 and 31 was the most frequent (53, 70.67%), followed by 16/33 (14, 18.66%) (Table 1).

According to the selection of samples at random, 89 HPV-positive samples were chosen to evaluate the cytological data and the respective viral type. The data are summarized in Table 2A and B. It was observed that when women were infected with HPV16, they had a higher incidence of low-degree intraepithelial lesion (LSIL; 30.34%) compared to high-degree intraepithelial lesion (HSIL; 16.85%). When the second most common viral type (HPV31) was considered, the results were different: 5.62% of patients presented HSIL and 3.37% LSIL. Patients presenting HPV33 showed no detectable lesions. Yet, these values were higher compared to the group showing the fourth most common viral type, HPV18, which showed 1.12% incidence of LSIL and HSIL. In the cases of multiple infection, the results showed HSIL in women infected with HPV16/31 (5.62%) and 16/31/33 (2.25%). For HPV18/31 the patients showed only HSIL (1.12%).

Table 2. A, B. Viral type and cervical lesion found in 89 samples.

A. Cervical alterations	Number of samples (%)
LSIL	38 (42.70%)
HSIL	29 (32.58%)
Carcinoma	22 (24.72%)
Total	89 (100%)
B. Cervical alterations/viral type	Number of samples (%)
LSIL	
HPV16	27 (30.34%)
HPV18	1 (1.12%)
HPV31	3 (3.37%)
HPV16/18	1 (1.12%)
HPV16/31	2 (2.25%)
HPV16/33	3 (3.37%)
HPV18/31/33	1 (1.12%)
HSIL	
HPV16	15 (16.85%)
HPV18	1 (1.12%)
HPV31	5 (5.62%)
HPV16/31	5 (5.62%)
HPV18/31	1 (1.12%)
HPV16/31/33	2 (2.25%)
Carcinoma	
HPV16	15 (16.85%)
HPV31	2 (2.25%)
HPV16/31	2 (2.25%)
HPV16/33	3 (3.37%)
Total	89 (100%)

HSIL = high-degree intraepithelial lesion; LSIL = low-degree intraepithelial lesion.

In patients presenting carcinoma, 16.85% had HPV16, followed by HPV31 (2.25%). Analyzing the simultaneous infections, 2.25% of patients had HPV16/31 and 3.37% had HPV16/33.

DISCUSSION

Uterus cervical cancer is the second most common type of cancer in females worldwide: 493,000 new cases every year and 50% mortality rate (Parkin and Bray, 2006; Rama et al., 2008).

In South America, Brazil has the highest incidence of cancer of the cervix, accounting for 50% of the 49,025 cancer cases registered in 2000 (INCA, 2007). Based on current rates of cervical cancer and demographic changes, 18,680 new cases of cancer of the uterine cervix are expected in 2008, corresponding to an estimated risk of 19.2 per 100,000 women. In different regions of Brazil, the incidence of the disease is heterogeneous, being 24.4 per 100,000 women in the South, 22.2 in the North, 19.4 in the Central West, 17.8 in the Northeast and Southeast. In Pernambuco, the expectation is 22.7/100,000 new cases and in Recife 25.6/100,000 in 2008 (INCA, 2007).

Considering the association HPV-cervical cancer, type 16 is the most frequent worldwide, with the exception of Indonesia where type 18 is the most common (Silva et al., 2006). Although HPV type 16 is also the most frequent in Brazil, the second most important type differs according to the Brazilian region. HPV types 31 and 33 rank below HPV type 16 in the northeastern and central-western regions of Brazil, whereas HPV type 18 is the second most frequent cervical cancer in the northern, southeastern and southern regions of Brazil (Silva et al., 2006).

The results presented here obtained in samples selected at random suggest that in the population of Recife, Pernambuco, HPV type 31 (15.49%) could have higher incidence compared with HPV type 18 (2.82%), and HPV type 33 shows similar incidence (2.82%).

Although HPV DNA is genetically stable, it should be considered if HPV infections are independent or if the different virus types can compete for the same tissue target. Some studies have shown that pre-existing cervical-vaginal HPV infections increase the acquisition risk of other HPV genotypes in females and males leading to the concept that this virus type competition is improbable. On the other hand, available data show that HPV-mediated infections are independent from each other and fail to prove that genotype substitution is probable (Woodman et al., 2007).

The current study showed a high percentage of simultaneous infection, HPV16/31 (70.67%) and HPV16/33 (18.66%). Previous reports have shown that concomitant or sequential detection of more than one HPV type is common and more frequent than expected (Mendez et al., 2005). If different HPV types compete for the cervical epithelium, the need for a generic type vaccination may increase (Mendez et al., 2005; Silva et al., 2006; Woodman et al., 2007).

Therefore, considering the present preliminary results, for the Recife population, the bivalent or quadrivalent vaccine, which are not effective against types 31 and 33, can lead to serious consequences. With a significant rate of simultaneous infection, as HPV16/31 and HPV16/33, the studies mentioned above indicate that the vaccination program has to consider the characteristics of the population.

The present analysis showed that women infected with HPV type 16 had a high level of LSIL (30.34%), higher than HSIL (16.85%), but women infected with HPV type 31 showed a higher level of HSIL (5.62%) compared to LSIL (3.37%). These results showing a higher level of HSIL associated with HPV31 have to be discussed, considering regional differences and eventual interaction with different co-factors.

When we analyze the lesion HSIL and simultaneous infection, the results show that women infected with HPV16 in combination with HPV31 (5.62%) have a higher level of

this lesion, when compared to women infected with HPV18/31 (1.12%) and HPV18/31/33 (2.25%). These data demonstrate that when the most prevalent HPV16 is combined with the second most common type, HPV31, the rate of more severe lesions increases.

Studying the level of LSIL in HPV16/33, we found 3.37% when compared with HPV16/31 (2.25%), indicating that the association of the first most common HPV in the population, the viral type 16, and the third most common, HPV33, can also increase the level of alterations.

Some researchers observed that women in southern Italy that were infected with HPV16 had high-grade intraepithelial lesions, but when they were infected by types 31 or 33 the frequency of HSIL was lower (14.3%) than if they were infected by types 16 (42.8%). Curiously, HSIL were not observed with the virus type 18 (Yalti et al., 2005).

In regard to the data obtained in women presenting carcinoma, women infected with HPV16 (16.85%) had greater incidence of carcinoma, followed by HPV31 (2.25%). In simultaneous infection, HPV16/31, carcinoma was detected in 2.25% of the women and HPV16/33 in 3.37%. Women infected by virus types 31 and 33, combined or not with other types, presented more lesions of the type HSIL, as compared to virus type 18, and these results again have to be evaluated considering environmental aspects.

The greatest challenge in public vaccination is the distribution of less expensive vaccines covering the most different or polyvalent HPV types. High-risk HPV types 31 and 33, which show different regional incidences, should be included in the vaccines (Yalti et al., 2005).

HPV types with variegated oncogenic potentialities will continue to cause clinically significant cervical lesions. Although current prophylactic vaccines protect non-infected females against viruses with great oncogenic potential, those infected prior to vaccination or infected with other virus types will continue to need diagnosis and treatment for pre-neoplastic lesions.

Results from the current study and from non-conclusive data on the efficiency of bivalent and quadrivalent vaccine for types 31 and 33 suggest that the available vaccines cannot meet the requirements of different populations, such as that of Recife. Some studies mention vaccine protection for the above-mentioned types, but nothing can be claimed regarding their efficaciousness when it has been found that types 31 and 33 are as frequent as or even more frequent than the vaccine's virus types.

Identification of HPV types most commonly associated with cervical cancer in different Brazilian regions should be taken into consideration regarding prevention strategies: more effective and polyvalent vaccines should be developed and produced (Coimbra et al., 2007; Góes et al., 2008; Beçak et al., 2009; Ruiz et al., 2009).

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