Article/Artigo

Human papillomavirus genotypes in asymptomatic young women from public schools in Rio de Janeiro, Brazil

Genótipos de papilomavírus humanos em mulheres jovens assintomáticas de escolas públicas do Rio de Janeiro, Brasil

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ABSTRACT

Introduction: The aim of this work was to survey HPV information from a random population of young women from Rio de Janeiro, Brazil. **Methods:** This cross-sectional study included cervical samples from 241 female students. To determine human papillomavirus status, polymerase chain reaction amplification was performed. HPV typing was determined by restriction fragment length polymorphism analysis. Demographic data, life style, sexual and gynecological history were obtained through use of a structured questionnaire. **Results:** The average age of the women was 19.6 years-old (SD=3.4 years). HPV prevalence was 27.4%. Nineteen different HPV genotypes were detected, including 13 high risk types. HPV 16 was the most prevalent type (6.2%), followed by 31 (4.1 %) and 66 (3.7%). Most of the oncogenic types belonged to the A9 species (28/48). The frequency of women infected by at least one oncogenic type was significantly higher than those only infected by low risk types (18.7% *versus* 7.5%). Cervical changes were detected in 12.5% of the sample and were significantly linked to infection with HPV types of the A9 species. Demographic variables, sexual initiation, or number of sexual partners were not associated with HPV prevalence, variety of HPV genotypes or oncogenic types. **Conclusions:** The relative frequency of HPV genotypes other than vaccine types in young females should be taken into account when evaluating vaccination strategies. Due to the high prevalence of HPV infection among the population studied, implementation of sex education in schools, promotion of condom use and an organized screening program to prevent cervical cancer must be encouraged for this age group.

Key-words: Human papillomavirus. Young women. Genotypes. Restriction fragment length polymorphism. Cohort study.

RESUMO

Introdução: O objetivo deste trabalho foi investigar a infecção por HPV em uma população randômica de mulheres jovens no estado do Rio de Janeiro, Brasil. **Métodos:** Este estudo incluiu amostras cervicais de 241 jovens. A detecção de papilomavírus humanos foi realizada pela reação da cadeia da polimerase e a tipificação deste vírus determinada pelo polimorfismo do comprimento do fragmento de restrição. Dados demográficos, estilo de vida, história sexual e ginecológica foram obtidos através de um questionário. A média de idade das mulheres foi 19,6 anos (SD=3,4). **Resultados:** A prevalência de HPV foi de 27,4% e 19 diferentes tipos foram detectados, incluindo 13 oncogênicos. O HPV 16 foi o tipo mais prevalente (6,2%), seguido do 31 (4,1%) e do 66 (3,7%). A maioria dos tipos oncogênicos pertencia à espécie A9 (28/48). A frequência de mulheres infectadas por ao menos um tipo oncogênico foi significantemente mais alta do que aquelas infectadas por tipos de baixo risco (18,7% versus 7,5%). Alterações cervicais foram detectadas em 12,5% das jovens e foram positivamente relacionadas a tipos de HPV da espécie A9. Variáveis demográficas, iniciação sexual e número de parceiros sexuais não foram associados com a prevalência de HPV, variedade de genótipos ou tipos oncogênicos. **Conclusões:** A frequência razoável de outros genótipos além dos vacinais nestas jovens deve ser considerada para avaliação de estratégias de vacinação. Devido à alta prevalência de HPV na população estudada, a implantação de educação sexual nas escolas, incentivo ao uso de preservativo e exames preventivos devem ser encorajados nesta faixa etária.

Palavras-chaves: Papilomavírus humano. Mulheres jovens. Genótipos. Polimorfismo do comprimento de fragmentos de restrição. Estudo de corte.

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INTRODUCTION

A large portion of sexually active women have been infected by one or more human papillomavirus (HPV) types of the genital area in their life time without causing any damage. However, certain HPV types, linked to behavior, demographic variables and inherited profile can induce severe cervical lesions or cancer in the infected host[1]. After their sexual debut, young women can present increased risk of acquiring the infection, which will mainly depend on the number of sexual partners[2]. Therefore, young girls have higher HPV prevalence than women above thirty years of age[3].

Despite the demographic differences in Brazil, some studies showed a general decrease in the first sexual intercourse in young women up to 1998, when a trend toward was established for an average of 14.9 years of age[3,4]. Early sexual debut increases the susceptibility of young women to sexually transmitted infections.

Estimates of HPV infection among young asymptomatic women vary from 19 to 31.4%[4,5,6]. The diversity of human papillomavirus covers over 100 genotypes, while distribution varies according to body tropism and oncogenic potential[7]. The prevalence of different HPV types varies according to geographic regions[10].

Several diagnostic assays permit detection of a large number of HPV types. Polymerase chain reaction (PCR) amplification followed by restriction endonuclease polymorphism assay can identify 44 types[8]. Natural infection does not confer group-specific immune protection or reinfection[9]; however, experimental studies show encouraging results for immune cross-reactivity among related high risk genotypes[10].

In the HPV vaccination era, it is useful to know the prevalence of low and high risk related types among female individuals at the age of vaccine administration. The goal of the current study was to survey information concerning HPV infection from randomly selected young women in the State of Rio de Janeiro, Brazil.

METHODS

Study population and study design

This prospective cross-sectional study was performed among 266 sexually active women aged from 14 to 26 years-old. The inclusion criterion was that all the referred women did not present prior history of cervical abnormalities. They were recruited from elementary and secondary public schools of Niterói City, Rio de Janeiro, Brazil between December 2004 and December 2005. All the subjects filled out a self administered questionnaire regarding their demographic data, life style, sexual and gynecological history.

Exclusion criteria

Students who were pregnant or presenting an immunosuppressive condition were excluded.

Specimen collection

Samples for routine Papanicolaou exam were collected after colposcopy examination. For the diagnosis of HPV DNA, additional samples were taken and placed in TRIS-EDTA buffered solution and frozen at -4°C until processed. According to the Bethesda nomenclature for cervical cytology, the cases were classified as: normal, ASCUS (atypical squamous cells of undetermined significance) and low grade squamous intraepithelial lesions (LSIL)[11].

Clinical and laboratorial data

The samples were incubated for 4 h at 50°C in digestion buffer (10mM tris-hydrochloric acid pH 8.3, 1mM EDTA pH 8.0, 0.5% Tween 20, proteinase K; at a final concentration of 400μg/ml). Later, they were extracted with phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated with one-tenth volume of 0.3M sodium acetate and three volumes of 100% ice-cold ethanol, washed with 70% ethanol, air-dried and suspended in 50μl of sterile water. For HPV detection, MY09/11 consensus primers were used, which amplify a 450-bp (base pair) within the L1 region of HPV (CGT CCM ARR GGA WAC TGA TC/ GCM CAG GGW CAT AAY AAT GG, where M = A + C, R = A + G, W = A + T, Y = C + T[12]. Amplification was conducted in 50μl of reaction mixture (1 X PCR buffer, 200mM dNTPs, 1.5mM MgCl2, 50pmol of each primer, 0.25U unit of Taq polymerase [Invitrogen Brazil, São Paulo, SP, Brazil] and 5μl of sample) with 35 amplification cycles. Each cycle included a denaturation step at 94°C for 1 min, an annealing step at 55°C for 2 min and a chain elongation step at 72°C for 2 min using DNA Thermal Cycler (Perkin Elmer, Cetus). Negative controls for background contamination received no DNA template. For HPV DNA detection, the DNA extracted from the samples was amplified with β-actin primers (0.1pmol each), amplifying a 330bp region of human DNA as an internal control[13]. The PCR product was analyzed on 1.3% agarose gel with ethidium bromide staining for visualization of DNA under UV light and molecular weight was determined by comparison with a 100-bp DNA ladder.

HPV typing was performed by restriction fragment length polymorphism analysis (RFLP) following PCR amplification. The 450 base pairs resultant from PCR products were digested by six restriction enzymes (BamHI, DdeI, HaeIII, HinfI, PstI, RsaI, Invitrogen, São Paulo, Brazil). The RFLP pattern of each sample was analyzed by agarose gel electrophoresis under UV light and compared to RFLP patterns for mucosal HPV[8]. The identified types were clustered in species-groups according to de Villiers et al, 2004[7]. Samples with coinfection were classified for high risk if they presented at least one high risk HPV type and as low risk if they presented only low risk HPV types.

Statistical analysis

A databank was generated in the SSPS-13 statistical packet. The statistical significance of the results was analyzed using the Chi square test. The level of significance was set at 0.05. To estimate HPV prevalence among students, we limited the analysis, *a priori*, to 250 students with 95% confidence interval. *A posteriori,* the strength of association between low risk/negative HPV was 9%; between normal, inflammatory/ASCUS, LSIL was 36%, and between nonA9/ negative, low risk HPV was 36%. For the remaining variables, the strength of association was above 90%.

Etical

Written informed consent was obtained from all participants and the study was approved by the Ethical Committee of the Medical College at the University.

RESULTS

Women were randomly selected from public schools in Niterói, Rio de Janeiro, Brazil. The overall sample consisted of 266 women who were asymptomatic for cervical lesions. Six of them were excluded from the analysis because their samples were inadequate for PCR amplification and one did not complete her clinical exams. The remaining 257 women included in the study were aged from 14 to 24 years-old, with a mean age of 19.6 years-old (standard deviation = 3.4 years). Fifty-six percent of the women were aged 14-19 years-old and 44%, 20-26 years-old. They attended high school (70.1%) or elementary school (29.9%). In the referred ethnicity, 70% were nonwhite. Most of the women did not smoke (83.3%) or drink (87.1%). Only 2.5% of the sample reported drug use. Sexual initiation prior to 15 years of age was reported by 55% of the women. Nearly 61% had had more than one life time partners and 38% of them had a stable partner at time of the study. Abortion episodes were reported by 12.8% and 66.7% had at least one child. Most of the women (87.5%) did not present cervical abnormalities. Among those presenting altered cytology, HPV alterations (1.9%), ASCUS (5%) and LSIL (5.4%) were observed. Constant condom use was declared by 9.4% of the students. No severe cervical lesions were identified.

Sixteen women who were HPV-positive for uncharacterized types were removed. Thus, the work focused on 241 eligible young women, among whom 66 (27.4%) were HPV-positive. Nineteen different HPV genotypes were identified, including 13 high risk types. The frequency of women infected by at least one oncogenic type was significantly higher than those infected by only by low risk types (20% *versus* 7.5%). The different HPV genotype results are presented in **Table 1**. Oncogenic HPV types from the A9 series were responsible for 12.4% of all infections, including coinfections (30/241). Type 16 was the most (6.2%) prevalent, followed by 31 (4.1%) and 66 (3.7%). The remaining types were observed at low frequencies. Among the low risk types, the A3 species were predominant, representing 5.4% of the types detected (13/241). Single infection was observed in 19.1% of the women, while 5.4% presented two or more HPV types. Vaccine types (6, 11, 16, and 18) were identified in 8.9% of infections (**Table 1**).

TABLE 1 - Prevalence of genital oncogenic and nononcogenic HPV types among students (N=241)

Species	High risk types	N	%
A9	16	15	6.2
	31	10	4.1
	52	3	1.2
	33	1	0.4
	58	1	0.4
A7	59	3	1.2
	18	2	0.8
	39	2	0.8
	82 subtype	1	0.4
A6	66	9	3.7
	53	5	2.1
	56	1	0.4
A5	26	1	0.4
	Total	**554**	**22.1**
Species	**Low risk types**	**N**	**%**
A15	71	5	2.1
A13	54	1	0.4
A10	11	4	1.7
	6	3	1.2
A3	61	4	1.7
	83	3	1.2
	84	2	0.8
	62	2	0.8
	81	2	0.8
	Total	**26**	**10.7**

TABLE 2 - Level of risk for HPV groups from 241 young women.

HPV groups	N	Cytology		OR	CI 95%	p
		Normal inflammatory	ASCUS LSIL			
Low risk	193	16(88.9)	2(11.1)	1.55	0.32- 0.52	0.57
Negative		162 (92.6)	13(7.4)			
High risk	241	33(68.8)	15(31.3)	5.31	2.40-12.08	<0.01
Negative or low risk		178(92.2)	15(7.8)			
Multiple infections	241	8(61.5)	5(38.5)	5.07	1.32-18.98	<0.01
Negative or low risk		203(84.0)	25(10.3)			
A9*	241	19(65.5)	10(34.5)	5.05	2.06-12.34	<0.01
Negative or low risk		192(90.6)	20(9.4)			
A9 excluding HPV 16	226	9(64.3)	5(35.7)	5.33	1.62-17.46	<0.01
Negative or low risk		192(90.6)	20(9.4)			
Non A9*	241	14(73.7)	5(26.3)	2.81	0.93-8.47	0.07
Negative or low risk		197(88.7)	25(11.3)			

* One woman carried two types of the same category.

ASCUS: atypical squamous cells of undetermined significance, LSIL: low grade squamous intraepithelial lesions, OR: odds ratio, CI: confidence intervals.

Common demographic variables and risk factors were not associated with the frequency of oncogenic or nononcogenic HPV. Since the average age was 19.6 years-old, this variable was divided into two ranges from 14-19 and 20-24 years of age. Both high and low risk HPV was randomly distributed in these two classes. The prevalence of multiple infections also showed a similar demographic and behavioral data distribution. To investigate the oncogenic HPV subset, cross tables with the same variables were constructed. The lack of association was maintained even when these were cross analyzed against the oncogenic A9 species.

Most of the women presented normal cytology. Women with abnormal cytology showed a significant frequency of oncogenic HPV infections {OR: 5.31 (2.40-12.08), p<0.01}. Cytological results versus A9 {OR: 5.05 (2.06-12.34), p<0.01} were markedly associated with abnormal specimens. In order to verify whether HPV 16 was driving this result, variables excluding this type were cross analyzed; the A9 group retained its significance (**Table 2**). Of the 13 multiple infections, 12 presented one oncogenic type, but clustering of HPV types involved both high and lower types indistinctly. Considering that most of the multiple infections involved at least one oncogenic type, the positive association between them and abnormal cytology was expected {OR 5.07 (1.32-18.98 p<0.01)} (**Table 2**).

DISCUSSION

In the current study, a random sample of young women recruited from public elementary or high schools of a metropolitan region was analyzed. The level of schooling indicates some knowledge concerning sexual information. On the other hand, most of the students had early sexual initiation and more than one sexual partner. However, these features were not associated with high HPV prevalence, the variety of HPV genotypes or the oncogenic types detected.

The women exhibited a wide spectrum of HPV genotypes. As expected for young women, oncogenic HPV types were predominant[4]. The A9 species, a particular phylogenetic group associated with oncogenesis, was observed in 12.5% of the whole sample. An American retrospective study involving 73,371 women described A9 types as having the highest likelihood of persistence[14]. According to Castle et al[15], when investigating a large random women's group, the prevalence of A9 types peaked in women aged less than 25 years-old, declined in middle ages and then increased slightly in older women.

The frequency of cervical alterations (12.3%) was expected in a young randomized sample[16]. The chance of women carrying abnormal cytology to be infected by a member of A9 species was significant (OR= 5.05). When HPV 16 was excluded from the study, the OR remained. This finding suggests that the A9 group, as a whole, could be as pathogenic as the HPV 16 type. This group was more pathogenic than non-A9 HPV types (OR=2.81).

HPV 18 is known as a prevalent type worldwide. It is the second most common high risk type associated with cervical cancer and the most strongly detected in cervical adenocarcinoma[17]. However, few cases harboring this type (0.8% of the total sample) were detected. Recent large surveys also reported the relatively low frequency of this type. Since HPV 18 has a rapid onset time for the development of cervical neoplasia, it is possible that its detection is more evident in high grade lesions[14,18].

Several uncommon types, such as the subtype 82, 39 and 59, classified as oncogenic types, but rarely associated with cervical cancers, were detected. Some authors consider them as intermediate

risk for cancer development[19]. According to the species classification described by Villiers et al[7], HPV 53 and 66 were categorized as high risk types. Even though they were detected in a relatively high frequencies, they were not associated with abnormal cytology. Some authors consider HPV 53, 56 and 66 as probable high risk types, belonging to the oncogenic A6 species[20]. However, HPV 53 was never observed in cervical cancer, having been verified at a relatively high frequency in normal smears, but rarely reported in cervical dysplasia. Other investigators have classified it as a low risk type[21]. Although the frequency of types varies according to region, some types spread worldwide. In a meta-analysis of HPV type distribution among women with HPV positive LSIL, the most common types were HPV 16, 31, 51, 53, 56, 52, 18, 66 and 58[22]. However, besides their highly variable geographic distribution and frequency, some of these types still do not have a defined pathogenic potential. In a work involving approximately 2,000 samples, 36% of unclear identifiable HPV infections were verified[23]. The clinical effects of these viruses cannot be determined according to their biological properties. Low risk types were detected in the women studied regardless of their cytology, suggesting the opportunistic character of these infections.

HPV coinfections have been observed more frequently among younger women[24]. Multiple infections with high risk types do not differ from single high risk infection regarding cervical dysplasia development[25]. Indeed, our data show a strong association between coinfections and the development of benign cervical abnormalities. Since most of them presented at least one oncogenic type, this was not a surprise.

The Brazilian National Agency of Health Vigilance (ANVISA) has approved two types of anti-HPV vaccines. Both are designed to protect against HPV 16, the most frequently oncogenic type. The target population includes women from 9 to 26 years, before they become sexually active. It is important to emphasize that 6.2% of the sample harbored HPV 16 and it was the only individual type clearly associated with abnormal results. Concerning the small size of the sample, this means that a reduction in HPV 16 incidence by prophylactic measures and current vaccines would greatly benefit young females. Nevertheless, other vaccine types were detected in a low prevalence and nonvaccine high risk types were determined in relatively high prevalences. It is possible that non-measured factors are contributing to alter HPV genotype frequency. The question concerning the coverage of all nonvaccine types widespread in the analyzed population remains unanswered, but some studies suggest cross-reactive immunity against other oncogenic nonvaccine types[26]. A polyvalent vaccine is currently in trials, including types verified in this study, such as HPV 31, 52, 58 and 66; however, immune interference, vaccine price and reactogenicity make it a difficult process. Anti-HPV vaccines are still under discussion[27,28]. Therefore, educational messages among young people are needed to understand the complexity of a vaccine against a sexually transmitted infection and cervical cancer[29]. Right now, screening programs and sex education are more relevant to prevent these diseases.

In summary, the high oncogenic HPV prevalence determined in this sample was not correlated to early sexual initiation or number of sexual partners. A9 oncogenic species as a whole was strongly observed in subjects carrying cervical lesions. Time and systematic surveillance will be necessary to prove whether or not well established oncogenic types infecting these women play a pathogenic role or whether they act as transient infections. At present, the implementation of sex education in schools, the promotion of condom use and organized screening programs to prevent cervical cancer must be encouraged for this age group.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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