Human papillomavirus genotype detection in recurrent respiratory papillomatosis (RRP) in Colombia

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ABSTRACT: *Background.* Knowledge on human papillomavirus (HPV) genotype distribution in recurrent respiratory papillomatosis (RRP) is essential to assess the impact of HPV vaccine. It is provided information for Colombia.

Methods. In all, 189 RRP primary cases diagnosed between 1985 and 2009 were identified from 5 pathology laboratories of Cali and Medellin, Colombia. HPV DNA testing in 129 cases that fulfilled inclusion criteria (available paraffin blocks, amplifiable DNA, and confirmed histologic diagnosis of RRP) was performed by the SPF-10/LiPA25 assay (version 1).

Results. Of all cases 36.1% were juvenile (<12 years old) and a majority of adults were males ($p = .09$); 95% of cases were HPV positive. HPV 6, 11, and 16 contributed to 69%, 27.1%, and 7.8% of all HPV positive cases. Twelve cases (9.3%) showed multiple infections; 8 of these were HPV 6 or 11 positive.

Conclusions. HPV prophylactic vaccine including HPV 6 and 11 may have a major impact against RRP. © 2012 Wiley Periodicals, Inc. *Head Neck* **35:** 229–234, 2013

KEY WORDS: recurrent respiratory papillomatosis, human papillomavirus 6, human papillomavirus 11, laryngeal papillomatosis, respiratory track infections

INTRODUCTION

Laryngeal papillomatosis or recurrent respiratory papillomatosis (RRP) is a benign neoplasm of the larynx that may extend toward the vocal cords, and may also affect the trachea and the lungs.[1] Its main clinical feature is the frequent recurrence after the surgical treatment of the lesion, and the difficulty to eradicate the papillomas from the respiratory tract, which leads to a high social and economic cost.[2] Incidences ranging from 0.24 to 4.3 cases per 100,000 children aged 14 years or younger have been reported for several geographic areas.[3–5] Although cases in all ages have been described, 2 age-related types have been described: the juvenile onset RRP (JORRP) occurs in children (mainly between 1 and 4 years of age) and adolescents, whereas the adult type occurs mainly in men between 20 and 30 years of age.[6] Their clinical complications include dysphonia, dyspnea, and, in serious cases, complete obstruction of the airways. Malignant transformation of the lesions has been described in approximately 3% to 7% of cases.[7] To date, there is not sufficient evidence to support the efficacy of antiviral agents as adjuvant therapy in the management of RRP.[8,9]

Human papillomavirus (HPV) is the etiologic agent of these lesions. In a review of 688 cases of RRP reported worldwide up to 1998, HPV DNA was detected in 524 cases (76.2%),[10] but other studies have reported prevalences of up to 100%.[11] The majority of these cases are benign squamocellular proliferations associated with genotypes 6 and 11, although HPV 16 has also been identified in a few lesions. HPV is involved in a variety of head and neck proliferative lesions, such as oropharyngeal cancer and sinonasal papillomas, in addition to being the main and necessary cause of cervical and other anogenital cancers.[12] The main hope to prevent the lesions caused by HPV resides today in the introduction of prophylactic

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HPV vaccines. Specifically, the quadrivalent vaccine (Gardasil®), which includes genotypes 11 and 6, may have a broad impact in the prevention of RRP. In Latin America, systematic information on the incidence and prevalence of RRP or information on the prevalence of HPV in these lesions is limited. The few studies reported from Brazil,[13] Chile,[14] Argentina,[15] Mexico,[16] and Venezuela[17] were marked by small sample size or lack of accuracy of the HPV DNA detection methods.

The purpose of this study was to identify the RRP cases that have occurred in the cities of Cali and Medellin in Colombia during the last 25 years and to determine their HPV DNA prevalence using a very sensitive polymerase chain reaction (PCR)–based assay. These data will be useful to estimate the impact that the introduction of the Gardasil® vaccine may have in preventing RRP.

MATERIALS AND METHODS

Study population and clinical cases

In all, 189 cases of RRP diagnosed between 1985 and 2009 were identified in the cities of Cali and Medellin. Cases from Cali were identified in the pathology laboratories of the 3 clinical centers (Imbanaco, University Hospital Universidad del Valle, and Fundacion Valle del Lili). Cases from Medellin were identified in 2 pathology laboratories located in the University Hospital San Vicente de Paul and in the Department of Pathology at the School of Medicine, University of Antioquia. Histologic slides of each case were recovered and used to confirm the histopathology diagnosis and the respective paraffin block of the confirmed cases were retrieved for further analysis. Basic information, such as sex, age, date of diagnosis, and location of injury, was obtained from medical records. Of 189 primary or incident RRP cases identified for demographic/clinical descriptive analysis, paraffin blocks were unavailable for 44, 1 paraffin block was unsuitable for microtome sectioning, 5 paraffin blocks did not include the RRP lesion, and DNA amplification was unsuccessful for 10 cases. Consequently, the final sample size for analysis of HPV genotype detection was 129 subjects (68% of identified cases).

Ethical consideration

This study was approved by the bioethics committee of the University Research Office at the University of Antioquia. Privacy, confidentiality, and anonymity were ensured in all procedures. This study followed the guidelines of the World Medical Association Declaration of Helsinki and the ethical principles for medical research involving human subjects contained in the International Ethical Guidelines for Epidemiological Studies prepared by the Council for International Organizations of Medical Sciences (CIOMS) in collaboration with the World Health Organization (WHO) for 2008.

Microdissection and DNA extraction

Five histologic sections were obtained from each paraffin block, wherein the first and the last sections stained with hematoxylin and eosin were used for histologic evaluation and the remaining sections for microdissection of the lesion. After histologic confirmation, lesions were circled and corresponding tumor areas microdissected from unstained slides using a sterile surgical scalpel in each case. The material obtained by microdissection was transferred to nonsilicone tubes and xylene (350 μL) was added to each sample to dissolve the paraffin. Paraffin-free tissue was precipitated with 150 μL of cold 100% ethanol and centrifugated; the pellet was allowed to dry at room temperature overnight. A dry button was resuspended in 100 μL of proteinase K buffer (10 mg/mL proteinase K in 50 mM Tris, pH 8.3) and incubated overnight at 37°C. Finally, the samples were incubated at 95°C for 8 minutes to inactivate proteinase K and stored at −20°C until use. Sectioning of paraffin blocks and DNA extraction were conducted under strict conditions to avoid contamination. Blank paraffin blocks with normal tissue were included among every 21 samples processed, and positive controls were paraffin-embedded cervical cancer tissues positive for HPV. DNA quality was evaluated by amplifying a 209 base-pair (bp) segment of the human β-globin gene.

Detection of HPV DNA

To identify HPV DNA, SPF-10 PCR[18] was conducted in a final reaction volume of 50 μL containing 10 μL of extracted DNA that was diluted 10-fold. The amplified PCR products were tested by use of hybridization, with a cocktail of conservative probes that recognized at least 54 mucosal HPV genotypes in a microtiter plate format for the detection of HPV DNA with a DNA enzyme immunoassay (DEIA). A microtiter plate reader was used to measure optical densities at 450 nm and the samples were classified as negative, positive, or borderline for HPV DNA. Borderline samples were rechecked by use of DEIA. After PCR, 10 μL of the amplimers that were positive for HPV DNA in the DNA immunoassay were used in the reverse hybridization line probe assay (LiPA25) (version 1; Laboratory Biomedical Products, Rijswijk, The Netherlands). LiPA25 can be used to detect 25 high-risk and low-risk HPV types (6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, and 74). Because SPF-10 PCR amplifies a DNA fragment of 65 bp, this technique has been widely shown to have high sensitivity to detect HPV DNA in paraffin-embedded tissue that has been stored for long periods.[19]

Data analysis

The percentage of cases positive for any HPV genotype, HPV-6, HPV-11, HPV-16, and other genotypes, was estimated among good-quality samples (β-globin positive even if they were HPV negative). A descriptive analysis of frequency of the epidemiologic characteristics of cases and HPV infection was conducted. Fisher's exact tests was used to test differences between the characteristics of HPV positive and negative or HPV 11 and 6 cases. The age was compared between HPV genotypes using the Kruskal–Wallis test. A significance level of 0.05 was used in all tests.

TABLE 1. Characteristics of individuals with recurrent respiratory papillomatosis of clinical centers of Cali and Medellin, Colombia.

Characteristic	No. (%) All cases $n = 189$	Included cases $n = 129$	p value*
Age at diagnosis, mean/SD, y	31.5/23.5	30/23.2	.91
≤4	30 (19.0)	23 (19.2)	
5–12	27 (17.1)	24 (20.0)	
13–34	23 (14.6)	18 (15.0)	
35–49	37 (23.4)	27 (22.5)	
≥50	41 (25.9)	28 (23.3)	
Missing values †	31	9	
Sex			.93
Female	74 (39.2)	50 (38.8)	
Male	115 (60.8)	79 (61.2)	
Sex in ≤12 years ($n = 57$)			.95
Female	27* (47.4)	22 (46.8)	
Male	30* (52.6)	25 (53.2)	
Sex in ≥13 years ($n = 101$)			.18
Female	33* (32.7)	24 (32.9)	
Male	68* (67.3)	49 (67.1)	
Acute inflammation‡			1.0
Absent	75 (54.0)	69 (53.5)	
Mild	39 (28.1)	36 (27.9)	
Moderate	16 (11.5)	16 (12.4)	
Severe	7 (5.0)	6 (4.7)	
Undetermined	2 (1.4)	2 (1.6)	
Chronic inflammation‡			.98
Absent	9 (6.5)	7 (5.4)	
Mild	85 (61.2)	81 (62.8)	
Moderate	39 (28.1)	36 (27.9)	
Severe	4 (2.9)	3 (2.3)	
Undetermined	2 (1.4)	2 (1.6)	
Dysplasia‡			1.0
Absent	52 (37.4)	49 (53.5)	
Mild	66 (47.5)	60 (27.9)	
Moderate	18 (12.9)	17 (12.4)	
Severe	1 (0.7)	1 (0.8)	
Undetermined	2 (1.4)	2 (1.6)	
Localization			.47
Supra glottic	52 (3.2)	1 (0.8)	
Glottic§	129 (81.6)	96 (81.4)	
Trachea	1 (0.6)	1 (0.8)	
Larynx (unspecified)	23 (14.6)	20 (16.9)	
Missing values	31	11 (8.5)	

NOTE: Values represent number (%) except as otherwise stated.
* Chi-square goodness of fit for included vs nonincluded cases; p value (chi-square test for sex distribution in <12-year-old vs >13 years old) = .09.
† Age at diagnosis could not be established in 17 males and 14 females.
‡ Histologic evaluation conducted in 139 cases with available paraffin blocks.
§ A case with lesion in the glottis and trachea was assigned to glottic.

RESULTS

Description of cases

Characteristics of the 189 primary cases as well as those of the 129 included in the analysis are described in Table 1. The mean age/SD of cases at diagnosis was 31.5/23.5 years (range, <1–77 years). It was not possible to retrieve information on age at diagnosis in 31 cases (14 females, 17 males). The majority of the cases (115/189, 60.8,%) were males. Among the 158 cases with information about the age, 57 cases (36.07%) were juvenile respiratory papillomatosis (≤12 years old) and 101 cases (63.9%) were adult respiratory papillomatosis (≥13 years old). Males accounted for the 52.6% and 67.3% of juvenile and adult cases, respectively. There was a higher frequency of males in cases of adult RRP, but the difference did not reach statistical significance (p value, chi-square = 0.09). In the histologic evaluation of 129 cases with available paraffin block and suitable for DNA analysis, all cases presented koilocytic changes compatible with HPV infection; approximately half of the samples did not show acute inflammation but the great majority (~94.6%) had some degree of chronic inflammation. Severe dysplasia was observed in 1 case and in the majority of cases (60.4%) mild and moderate dysplasia was observed. A total of 81.6% of cases were lesions on the glottis and 14.6% in the larynx. No significant statistical differences were observed in the age, sex, and site distributions, in the degree of inflammation and dysplasia either between the entire group of 189 or the study group of 129.

HPV genotyping

In 10 of the 139 cases with available paraffin block, there was not adequate DNA for amplification (β-globin and HPV negative). Table 2 shows the results of HPV genotype distribution in 129 cases of RRP. The overall prevalence of HPV DNA in RRP was 95.3%. Eighty-nine samples (69%) were positive for HPV 6, 35 (27.1%) for HPV 11, and 10 (7.8%) for HPV 16. There were 12 cases with coinfections, 7 of which were infected with HPV 6 and 11 and 2 with HPV 6 and 16. Two other samples were coinfected with HPV 6 and 52 or 54. In one sample HPV 6, 11, 16, 39, and (68 or 73), were simultaneously identified (data not shown).

There were no statistically significant differences in the distribution of HPV 6 or 11 for age and sex ($p > .05$, Fisher's exact test), as well as for severity of inflammation, degree of dysplasia, and localization of the lesions.

DISCUSSION

RRP is a very rare, potentially life-threatening clinical condition. The majority of cases require multiple surgeries during the entire life, to remove the recurrent papillomas and, in some cases, poor prognosis is due to inaccessibility of the distal pulmonary disease and tracheal involvement.[1] Currently, there are no therapeutic measures that can afford complete remission.[8,9] RRP is caused by members of the Papillomaviridae family and prevention of the disease may rely on the recently introduced quadrivalent vaccine (Gardasil®) that can prevent infections of HPV 11 and 6,[20] the most frequent genotypes found in this type of lesion. To determine the impact of the vaccines in preventing RRP, accurate estimates of HPV prevalence on the disease are needed. In Latin American there are very few reports of surveys describing the distribution of HPV in RRP. Here we report the prevalence of HPV in RRP cases diagnosed between 1985 and 2009 in 5 pathology laboratories that serve 4 clinical centers of 2 main cities of Colombia. Compared with other studies conducted in Latin America,[13–17] this study reports the highest number of consecutive cases of RRP during a 25-year period. The demographic characteristics of the cases are similar to the previously described data.[6]

TABLE 2. Prevalence of HPV in individuals with recurrent respiratory papillomatosis of clinical centers of Cali and Medellin, Colombia.

Characteristic	No. (%)				
	Cases	HPV positive	HPV-6	HPV-11*	HPV-16
Total[†]	129	123 (95.3)	89 (69.0)	35 (27.1)	10 (7.8)
Coinfections	12 (9.3)	12 (9.8)	12 (13.5)	8 (22.9)	3 (30.0)
Ages, mean/(SD), y	30/(23.2)	29.3/(22.7)	29.2/(22.2)	31.7/(25.5)	35.1/(24.6)
≤4	23 (19.2)	22 (95.7)	15 (65.2)[§]	6 (26.1)	2 (8.7)[§]
5–12	24 (20.0)	23 (95.8)	17 (70.8)[¶]	6 (25.0)[¶]	1 (4.2)
13–34	18 (15.0)	18 (100.0)	14 (77.8)	4 (22.2)	0 (0.0)
35–49	27 (22.5)	26 (96.3)	19 (70.4)[‖]	5 (18.5)[‖]	4 (14.8)
≥50	28 (23.3)	25 (89.3)	18 (64.3)**	9 (32.1)**	2 (7.1)**
Missing values[‡]	9	9	6[††]	5[††]	1
Sex in ≤12 years ($n = 47$)					
Female	22 (46.8)	20 (90.9)	13 (59.1)	8 (36.4)	0 (0.0)
Male	25 (53.2)	25 (100.0)	19 (76.0)	4 (16.0)	3 (8.7)
Sex in ≥13 years ($n = 73$)					
Female	24 (32.9)	22 (91.7)	15 (62.5)	7 (29.2)	2 (8.3)
Male	29 (67.1)	47 (95.9)	36 (76.6)	11 (22.4)	4 (8.2)
Acute inflammation					
Absent	69 (53.5)	65 (94.2)	49 (71.0)	14 (20.3)	6 (8.7)
Mild	36 (27.9)	34 (94.4)	24 (66.7)	10 (27.8)	1 (2.8)
Moderate	16 (12.4)	16 (100.0)	9 (71.0)	9 (56.2)	2 (8.7)
Severe	6 (4.7)	6 (100.0)	5 (83.3)	1 (16.7)	1 (16.7)
Undetermined	2 (1.6)	2 (100.0)	2 (100.0)	1 (50.0)	0 (0.0)
Chronic inflammation					
Absent	7 (100.0)	6 (85.7)	0 (0.0)	1 (14.3)	7 (100.0)
Mild	78 (96.3)	57 (70.4)	23 (28.4)	5 (6.2)	78 (96.3)
Moderate	33 (91.7)	21 (58.3)	9 (25.0)	4 (11.1)	33 (91.7)
Severe	3 (100.0)	3 (100.0)	2 (66.7)	0 (0.0)	3 (100.0)
Undetermined	2 (100.0)	2 (100.0)	1 (50.0)	0 (0.0)	2 (100.0)
Dysplasia					
Absent	47 (53.5)	36 (73.5)	12 (24.5)	5 (10.2)	47 (95.9)
Mild	57 (27.9)	40 (66.7)	15 (25.0)	5 (8.3)	57 (95.0)
Moderate	17 (12.4)	11 (64.7)	7 (41.2)	0 (0.0)	17 (100.0)
Severe	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Undetermined	2 (100.0)	2 (100.0)	1 (50.0)	0 (0.0)	2 (100.0)

Abbreviation: HPV, human papillomavirus.
* p value of Fisher's exact test = .044 for HPV 11 status versus acute inflammation.
[†] Includes only adequate samples (β-globin or HPV positive).
[‡] Age at diagnosis could not be established in 4 females and 5 males.
[§] One sample has multiple infection with HPV 6 and HPV 16.
[¶] One sample has multiple infection with HPV 6 and HPV 11.
[‖] Two samples have multiple infection: 1 was HPV 6 and HPV 11 and the other with HPV 6 and HPV 16.
** Five samples has multiple infection: 2 were HPV 6 and HPV 11; 1 was HPV 6 and HPV 52, and 1 was HPV 6 and HPV 54. One sample was positive for HPV 6, 11, 16, 39, 68, or 73.
[††] Three samples have multiple infection with HPV 6 and HPV 11.

There was a bimodal age distribution and in adults (≥13 years) a predilection for males (Table 1). We were able to conduct HPV detection in 129 cases and the exclusion of cases because lack of paraffin blocks did not change the demographic characteristics of the cases. The use of a standard protocol for identification and microdissection of the lesions and use of a very sensitive PCR-based assay, especially suitable for paraffin-embedded tissue,[21] further strengthens our findings.

In this study, 95% of samples were HPV positive and HPV 6 was detected in 69%, HPV 11 in 27.1%, and HPV 16 in 7.8% of samples. Only 9.3% of samples showed multiple infections, and HPV 16 was present with either HPV 11 or HPV 6 genotypes in 2.3% of the samples. Studies conducted around the world have reported HPV DNA in almost 100% of cases with HPV 6 and 11 in virtually 90% of them and similar rates of multiple infections.[11] Table 3 summarizes the studies so far reported from Latin America.[13–17] Results of the study conducted in Brazil[13] reflect our findings. In a study conducted in Mexico[16] HPV was detected in 100% of the samples, 74% showed coinfection with 2 or more viral types, and HPV 16 was identified in 83% of them. HPV 6 and HPV 11 were identified in only 70% of these cases and in all except a single sample, these genotypes were identified along with high-risk HPV types 16, 31, 33, 35, and 39.

The discrepancy in the Mexican study and our results could be explained by PCR contamination as a result of overmanipulation of the samples during the 2 rounds of PCR. In the study conducted in Venezuela,[17] HPV DNA was detected in 53% of the samples and only 27% of them were HPV 6 or HPV 11 positive. In cases from Argentina[15] all samples were HPV positive but HPV 6 or HPV 11 DNA was detected in only 58% of the cases. In the above-mentioned studies various PCR assays were

TABLE 3. Prevalence of HPV in individuals with recurrent respiratory papillomatosis in Latin American studies.

Author	Country	HPV detection method	HPV prevalence		HPV 6 (%)	HPV 11 (%)	Coinfections (%)
			n	(%)			
Levi et al 1989[13]	Brazil	PCR MY09/11	19	19/19 (100)	14/19 (74)	3/19 (16)	1 (5)
Rico et al 1993[14]	Chile	ISH	8	7/8 (87.5)*	3/8 (37.5)		ND
Gomez et al 1995[15]	Argentina	PCR MY09/11/nested GP5/6	12	12/12 (100)	7/12 (58.33)		ND
Penaloza-Plascencia et al 2000[16]	Mexico	PCR CPI/CPIIG (EI)	47	47/47 (100)†	15/47 (32.0)	18/47 (38)	35/47 (74.4)
Bello de Alford and Caibe 2001[17]	Venezuela	PCR (primers not reported)	15	8/15 (53)	4/15 (26.6)	4/15 (26.6)	ND
Sanchez et al 2011§	Colombia	PCR-RLB	129	123/129 (95.3)	89/123 (72.3)	35/123 (28.4)	12/123 (9.7)

Abbreviations: PCR, polymerase chain reaction; ISH, in situ hybridization; ND, not detected; HPV, human papillomavirus.
* Other HPV genotypes found in this study were: HPV 16 or 18 (4/8, 50%).
† Other HPV genotypes found in this study were: HPV 16 (39/47, 83%), HPV 31 (2/47. 4%), HPV 33 (26/47. 55%), HPV 35 (13/47. 28%), and HPV 39 (7/47, 15%).
§ Data obtained in this study. Other HPV genotypes found in this study were: HPV 16 (10/129, 7.8%), HPV 52, 54, 39, 68, or 73 were present once with either HPV 6 or 11. Prevalence of genotypes estimated among HPV-positive samples.

used and only juvenile papillomatosis cases were included. In our study the HPV positivity rate was similar in juvenile and adult onset cases and, although there was a higher percentage of girls <12 years old positive for HPV 11, there was no statistically significant difference of HPV 11 and HPV 6 distribution by sex and by age. It has been shown that different methods have different sensitivities for specific genotypes, especially when paraffin-embedded material is used.[22]

Variability in the methodology used for HPV testing may account for the differences observed among the Latin American studies. It was estimated that the 3 main pathology laboratories in Cali that provided cases for our study process about 60% of the total of cases diagnosed in this city, whereas in Medellin, the 2 pathology labs process about half of the cases diagnosed in that city. Therefore, our study population is not entirely representative of the total population of RRP cases diagnosed in the 2 cities. Another possible limitation in the data is that HPV DNA was detected in paraffin-embedded blocks of tissue that have been stored for long periods of time. However, the low rate of inadequate samples and high rate of HPV positivity in adequate samples suggest this did not represent a source of bias in the frequency of HPV types detected. The knowledge of the predominant role of HPV 6 and HPV 11 in cases of RRP of Colombia confirm the current availability of opportunities for 2 main strategies of prevention of this disease. First, prophylactic HPV vaccine that includes HPV 6 and HPV 11 is highly efficacious in preventing diseases related to these genotypes.[20] This would reduce the HPV genital infections and subsequent lesions caused by types 6 and 11 in future mothers, with a likely subsequent additional benefit of reducing RRP incidence among children of these future mothers.

Previous studies in Cali have shown that HPV 16 and HPV 18 account for approximately 65% to 70% of cervical cancers and HPV 6 and HPV 11, although rare in these cancers, account for 5% to 10% of women with low-grade squamous intraepithelial lesion (LSIL) or normal cytology.[23,24] In Medellin, the overall HPV prevalence in women with normal cytology is 10.2%; the most common genotypes observed are HPV 16 (2.3%), HPV 18 (1.7%), and HPV 56 (1.4%). HPV 6 and HPV 11 account for 0.16% and 0.24% of infections, respectively.[25] Second, recent communications suggest prevention of recurrences of RRP with human papillomavirus vaccination.[26,27] This observation warrants furthers studies evaluating the efficacy of HPV vaccine to prevent recurrences during the time course of the disease.

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