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HUMAN PAPILLOMAVIRUS RISK FACTORS FOR INFECTION AND GENOTYPE DISTRIBUTION IN ABORIGINAL WOMEN FROM NORTHERN ARGENTINA

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Abstract The mortality rate for cervical cancer (CC) in Northern Argentina is three times higher than the average for the country (7.8 deaths/100 000 women). We determined the prevalence and genotype distribution of human papillomavirus (HPV) in 227 sexually active women of the native *Pilagá* community in Formosa, Argentina. We also conducted an HPV-16 variant analysis and studied several community factors that might play a role in viral entry and infection. Endo- and exocervical samples were tested for HPV DNA with MY09/11-PCR or with GP5+/6+-PCR. HPV was detected in 46.7% of the samples and 21 different types were found; the most frequent being HPV-16 (19.4%), -6 and -18 (5.3%), -58 (3.5%) and -31 and -33 (3.1%). In relation to HPV-16 variants, 68.2% were European and 31.8% Asian-American. Among the cofactors analyzed only disposal of human excreta to the open air (P=0.01) was significantly associated with HPV infection. Our prevalence estimates clearly show that *Pilagá* women are highly exposed to or infected with high risk HPV types and therefore are at a high risk of developing precancerous lesions and eventually CC at the population level.

Key words: aboriginal women, human papillomavirus infection, viral sexually transmitted disease

Resumen ***Virus papiloma humano (HPV) en mujeres de la etnia Pilagá del nordeste argentino: Factores de riesgo de infección y distribución de tipos virales.*** La tasa de mortalidad por cáncer cervical (CC) en la región norte de la Argentina es tres veces más alta que la media del país (7.8 muertes/100 000 mujeres). En el presente trabajo se determinó la prevalencia de infección por virus papiloma humano (VPH) y la distribución y frecuencia de los genotipos en 227 mujeres sexualmente activas de la etnia aborigen Pilagá (Formosa, Argentina). También se realizó un análisis de las variantes intratípicas de VPH-16 presentes en la comunidad y se analizaron diversos factores socioculturales que podrían tener algún rol destacado en la transmisión de la infección viral. Se estudiaron muestras de células endo-exocervicales mediante PCR basadas en los cebadores MY09/11 y GP5+/6+ con posterior restricción enzimática y/o hibridación *dot-blot*. La infección por VPH fue detectada en el 46.7% de las mujeres analizadas. Fueron identificados 21 genotipos, de los cuales los más frecuentes fueron HPV-16 (19.4%), -6 y -18 (5.3%), -58 (3.5%) y -31 y -33 (3.1%). Respecto al HPV-16, se encontraron 68.2% de variantes europeas y 31.8% de asiático-americanas. Entre los cofactores analizados, solo la disposición de excretas al aire libre estuvo significativamente asociada con la infección por VPH (P = 0.01). Los datos obtenidos reflejan que la comunidad Pilagá está altamente expuesta a las infecciones por genotipos de alto riesgo de VPH, lo cual puede estar asociado a una alta incidencia de lesiones cervicales preneoplásicas y neoplásicas.

Palabras clave: mujeres aborígenes, infección por virus pailoma humano, enfermedad viral de transmisión sexual

Human papillomavirus (HPV) infection is known to cause cervical cancer (CC)[1, 2]. More than 120 HPV types have already been characterized, but only the high-risk (HR-HPV) types are considered as true human carcinogens[3]. Within this group, HPV-16 and -18 are the most prevalent viral types associated with CC, accounting for more than 65-70% of cases worldwide[4, 5].

Argentina is characterized by the multi-ethnic composition of its population. This particular situation explains the existence of large disparities in both socio-economic development and cultural characteristics, including sexual behaviour among communities from different regions

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of the country. Understanding how these features relate to the acquisition of HPV infections is important in order to identify subgroups that are at higher risk for CC[6]. The annual mortality rate for CC in Argentina is 7.8/100 000 women, but this rate is three times higher in the North Eastern region of the country, where a wide spectrum of Indian and isolated white communities live in unfavourable conditions in terms of socioeconomics and health[7].

The aim of our study was to estimate the HPV prevalence in the Pilagá Indians, a native community from Northern Argentina, and to carry out HPV typing, HPV-16 variant analysis and the study of several community factors that might play a role in viral entry and infection.

Materials and Methods

A cross-sectional community-based study was conducted between March 2007 and March 2009 among 235 women aged 13 to 68 (mean age 30 years) of the Pilagá aboriginal ethnia. After excluding samples that were not suitable for cytological and virological analysis, 227 women were finally included in this study, representing 20% of the total sexually active women in the community.

A standardized questionnaire was used to interview the participants regarding their clinical history, sexual behavior, cultural habits and socio-economic and living conditions. Confidentiality was guaranteed for all participants to ensure as frank and complete answers as possible. After providing informed consent, all women were examined by a gynecologist.

The study was reviewed and approved by the Ethics Committee of the *Instituto de Medicina Regional* of Universidad Nacional del Nordeste (Argentina) in accordance with the Helsinki Declaration.

Exfoliated cervical cells were collected from all women by sampling the ecto- and endocervix using a wooden spatula for Papanicolau smears (PAP) and a cytobrush for HPV DNA detection. Cervical cells from the latter were eluted in sterile phosphate-buffered saline (PBS) contained in a tube, and then transported on ice to the laboratory. Upon arrival at the lab, and after checking the sample container's integrity, the cells of each sample were pelleted by centrifugation and kept frozen at -70 °C until processing.

DNA was obtained by treating pellets with 400–700 µl of homogenization solution (2% cetyltrimethylammonium bromide,1.4 M NaCl, 0.2% β-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl pH 7.5), extracting the DNA using chloroform:isoamyl alcohol (24:1), then precipitating with absolute ethanol and resuspending in 50-100 µl of sterile bidistilled water. All samples were checked to assess the quality and integrity of the DNA by amplifying a known region of the human β-globin gene (268 bp). Samples that were negative for the β-globin test were discarded.

PAP smears were processed and analyzed according to the 2001 Bethesda Classification System. The final diagnosis was based on the worst morphological picture.

HPV DNA detection and typing was conducted using the widely known MY09/11 polymerase chain reaction (PCR) (450 bp PCR product) followed by the restriction fragment-length polymorphisms (RFLP) technique, as previously described[8, 9]. Briefly, the products of MY09/11 PCR were first electrophoresed on a 2% agarose gel, stained with ethidium bromide and visualised under UV light. Then, the amplicons of positive samples were digested by seven restriction enzymes (BamHI, DdeI, HaeIII, HinfI, PstI, RsaI and Sau3AI) in individual microtubes. After that, the RFLP product of each sample was electrophoresed on a 3% agarose gel, stained with ethidium bromide, and photographed under UV light. Each restriction pattern was compared with published data to identify the genotype(s) involved.

HPV-positive samples that were difficult to analyze by PCR-RFLP either due to the presence of nonspecific amplimers or weak amplicon signals on agarose gels, were reanalyzed by PCR using GP5+/6+ primers followed by dot-blot hybridization with type-specific oligonucleotide probes as previously described[10]. Probes for HPV types 6, 11, 16, 18, 31, 33 and 45 were used. The bound probes were detected with streptavidin-horseradish peroxidase and enhanced chemiluminescent substrate (*Amersham ECL*™ Systems). Samples that remained untyped were classified as undetermined HPV types (HPV-X).

To determine the genotype variants of the HPV-16-positive samples, a 364-bp segment (nucleotide positions 7478 to 7841) of the long control region (LCR) was analyzed by PCR-sequencing according to the method described by *Ho* et al[11]. The LCR sequencing reaction was performed using the Big Dye-terminator 3.1 chemistry, and DNA sequencing was conducted using the Applied Biosystems Division Automated 3100 DNA Analyzer.

All completed interviews were carefully reviewed by the researchers for completeness prior to entering data into computer files. Continuous variables were categorized, and the risk associated with HPV positivity was calculated for each category of a variable, adjusted by age. Univariate statistics were calculated for all variables, and multivariate logistic regression analysis was performed in order to identify the independent variables that influence the relative risk of HPV positivity (95% CI). The limit of statistical significance was set at $p<0.05$. Data were analyzed using the Epi-Info™ 3.5.1 Statistical Program (Centers for Disease Control and Prevention, Atlanta, GA).

Results

Out of the 227 women included in the study, 36 (15.8%) had negative PAP smears for squamous intraepithelial lesion (SIL) or malignancy, 173 (76.2%) showed reactive cellular changes (RCC) (mainly due to inflammation), 16 (7%) low-grade SIL (LG-SIL), 1 (0.4%) high-grade SIL (HG-SIL), and 1 (0.4%) CC. HPV DNA was detected in 46.7% of the samples (106/227 women) with statistically significant differences in terms of positive results according to the cytological category; the prevalence of HPV was estimated as 27.8% in women with a normal PAP test, 46.2% in women with RCC, 87.5% in women with LG-SIL and 100% in women with HG-SIL and CC (P=0.01). Table 1 shows the results of the detection of HPV DNA and the distribution of the positive results according to the risk grade of the HPV and cytological category.

The socio-economic evaluation of the living conditions of the *Pilagá* community showed that the majority of the participants had completed elementary school education (76.8%), but only 27.5% had a formal or informal occupation at the time of the study; 98.3% had

TABLE 1.– *HPV infection stratified by grade of cervical status among sexually active women of the Pilagá community*

Cytology[a]	N (%)	HPV Positive %)	p	HR-HPV[f] Positive (%)	p
Negative for SIL or malignity	36 (15.8)	10 (27.8)	0.01	7 (19.4)	0.01
RCC[b]	173 (76.2)	80 (46.2)		50 (28.9)	
LG-SIL[c]	16 (7)	14 (87.5)		12 (75.0)	
HG-SIL[d]	1 (0.4)	1 (100)		1 (100)	
CC[e]	1 (0.4)	1 (100)		1 (100)	
TOTAL	227 (100)	106 (46.7)		71 (31.3)	

[a]*According to 2001 Bethesda System;* [b]*Reactive cellular changes. Subcategory of negative for SIL or malignity;* [c]*Low-grade squamous intraepithelial lesion;* [d]*High-grade squamous intraepithelial lesion;* [e]*Cervical cancer;* [f]*high-risk HPV type.*

no sanitary latrines in their houses and only 5% had well water for drinking and cleaning. Two logistic regression models generated prevalence odds ratio estimates of HPV DNA detection associated with each socioeconomic, behavioural and sexual characteristic. One model was adjusted for age effects and one was further adjusted for all variables considered as possible confounders (data not shown). We were unable to evaluate the impact of tobacco or the use of oral contraceptives because only a small proportion of women referred to a smoking habit (3.1%) and less than 6% of the women had used oral contraceptives at some point in their life. There was no association between age, age at first intercourse, number of lifetime sexual partners, number of pregnancies, level of literacy, employment and the risk of HPV infection. However, we found a significant correlation between HPV-positive results and the disposal of human excreta to the open air (41/71 = 57.5%) instead of using sanitary latrines (64/156 = 41.7%; OR: 2.38, CI 95% = 1.19-4.75, P = 0.01).

The individual prevalence of the 21 detected HPV types is presented in Table 2. Overall, the most frequent HPV types were HPV-16 (19.4%), -6 and -18 (5.3%), -58 (3.5%) and -31 and -33 (3.1%). Multiple HPV infections were observed in 28 cases (12.3%), including 24 cases with two viral types and 4 cases with three different genotypes. Out of them, 25 multiple infections involved one or more high-risk HPV types.

In relation to HPV-16, 22 positive cervical samples were examined to determine the genomic variants of this viral type. Of these, 7 (31.8%) were Asian-American (AA) variants and 15 (68.2%) were European (E) (Table 3).

Discussion

Only a few epidemiological studies have estimated HPV prevalence, HPV type distribution and the risk factors associated with HPV infection in South American Indian populations[12-16]. However, these communities constitute a substantial proportion of the population in certain regions of Latin America, as in Northern Argentina. Furthermore, the particular conditions of aboriginal communities in terms of migration, social exchange and specific sexual habits can have an important impact on public health, mostly due to interactions with the inhabitants of nearby regions. Despite the numerous logistical and cultural barriers that have hindered our work in the *Pilagá* population, we were able to recruit and study 20% of the sexually active women in this community.

The cervical cytology study showed that 92.1% of the women analyzed had a normal PAP test; however, a high proportion of this cytological category (82.8%) showed inflammatory changes, mainly due to bacterial and/or parasitic infection (data not shown). This situation is very common among aboriginal communities in Northern Argentina, and mainly reflects their almost non-existent access to healthcare[6, 13, 14].

The worldwide prevalence of HPV infection ranges from 2 to 44% in the general population and, on the whole, developing countries seem to have higher rates[4,17]. A consistent worldwide meta-analysis on global HPV prevalence showed an infection rate of 10.4% for the world and 12.9% for Latin America, HPV-16 being the most common type in both normal and pathological populations[17]. In our study, the overall prevalence of HPV was very high (46.7%), similar to the values found in developing countries having serious problems in implementing and maintaining a sustainable gynecologic health policy[18,19]. It is also comparable with the results obtained by other authors for other indigenous populations of Northern Argentina (prevalence ranging from 52% to 60%)[12, 13], but clearly exceeds the mean frequency for populations from the central and southern regions of the country and for Latin America[4, 17, 20]. There was no trend pattern between HPV prevalence and age. Indeed, HPV prevalence in this community was high in all age groups (around 30–60%), and the customary peak among young women, as well as the steady decrease among middle-aged women, described in a number of regions, was not observed in this population[21, 22]. Accordingly, a lack of a decrease of HPV with age was previously observed in regions with an overall high HPV prevalence[23-25].

In relation to the genotypes found, the high-risk HPV types were present in 67% of all infected women, and HPV-16 clearly stood out among the 21 different types identified in this work, consistent with worldwide data

TABLE 2.– Prevalence of 21 HPV types by cytological findings and overall among 227 Pilagá women, Northern Argentina

HPV type[a]	Cytology results						All women studied	
	Normal (n = 36)		RCC (n = 173)		≥L-SIL (n = 18)		(n = 227)	
	N	%	N	%	N	%	N	%
Any HPV	14	38.9	103	59.5	21	117	106	46.7
HR-HPV								
16	5	13.9	29	16.8	10	55.5	44	19.4
18	2	5.5	10	5.8	0	0	12	5.3
31	2	5.5	5	2.9	0	0	7	3.1
33	1	2.8	5	2.9	1	5.5	7	3.1
35	0	0	3	1.7	0	0	3	1.3
45	0	0	2	1.2	1	5.5	3	1.3
58	0	0	5	2.9	3	16.7	8	3.5
Any HR-HPV	10	27.8	59	34.1	15	83.3	84	37
Probable HR-HPV								
26	0	0	1	0.6	0	0	1	0.4
53	2	5.5	4	2.3	0	0	6	2.6
66	1	2.8	2	1.2	2	11.1	5	2.2
Any probable HR-HPV	3	8.3	7	4	2	11.1	12	5.3
LR-HPV[b]								
6	0	0	11	6.4	1	5.5	12	5.3
11	0	0	4	2.3	2	11.1	6	2.6
40	0	0	1	0.6	0	0	1	0.4
54	0	0	1	0.6	0	0	1	0.4
61	0	0	5	2.9	0	0	5	2.2
62	0	0	1	0.6	0	0	1	0.4
70	0	0	2	1.2	0	0	2	0.8
71	0	0	1	0.6	0	0	1	0.4
81	0	0	1	0.6	1	5.5	2	0.8
84	0	0	5	2.9	0	0	5	2.2
102	0	0	1	0.6	0	0	1	0.4
Any LR-HPV	0	0	33	19.1	4	22.2	37	16.3
HPV-X[c]	1	2.8	4	2.3	0	0	5	2.2
Single infections	6	16.7	61	35.3	11	61.1	78	34.4
Multiple infections	4	11.1	19	11	5	27.7	28	12.3
Groups of types[d]								
16/18	7	19.4	39	22.5	10	55.5	56	24.7
16/18/6/11	7	19.4	54	31.2	13	72.2	74	32.6
16/18/31/45	9	25	46	26.6	11	61.1	66	29.1

[a]*Sum of distribution percentages exceeds the total number of prevalence because, in the numerator, women with multiple infections are counted in each of the HPV types found.* [b]*Includes HPV-types of unknown oncogenic risk (HPVs 62, 71, 84, 102).* [c]*HPVs that could not be typed.* [d]*HPV-types covered by licensed vaccines; the percentages displayed in this section can be taken as the effective coverage of vaccines only under the assumption of full cross-protection between HPVs 16/18 and 31/45, which remain to be determined.*

and with previous studies on different populations in Argentina[4, 17, 12, 13]. In addition, the four HPV types most prevalent in *Pilagá* women (HPV-16, -18, -6, and -11) represented 70% of all infections detected and 32.6% of the total number of women analyzed. These are very important baseline data for estimating the theoretical impact of the HPV vaccine and for planning future post-vaccination epidemiological surveillance.

TABLE 3.– Nucleotide substitutions identified in the LCR[a] sequence of European and non-European variants compared to HPV-16 reference sequence

Variant	Class/subclass	n	Changed nucleotidic positions in LCR									
			7485	7489	7507	7521	7669	7689	7729	7743	7764	7786
E[b]	Reference sequence	-	A	G	A	G	C	C	A	T	C	C
E	E (G-11)	5	-	-	-	-	-	-	-	-	-	-
E	E (G-1)	10	-	-	-	A	-	-	-	-	-	-
AA[c]	AA (IND-8)/a	2	C	A	-	A	T	A	C	G	T	T
AA	AA (B-14)/c	5	C	A	G	A	T	A	C	-	T	T

[a]*Long Control Region.* [b]*European.* [c]*Asian-American*

The proportion of infections by multiple HPV types was within the expected range, most of them involving high-risk types (87.5%) and being more frequent in PAP smears with RCC and L-SIL+ than in normal ones. Other studies have shown a similar tendency[26], and since the implication of HPV infection with multiple genotypes remains unclear for CC development, it is important to accumulate epidemiological information for further analysis[27, 28].

Among the risk factors analyzed in the *Pilagá* community for HPV infection, the disposal of excreta in the open air was found to be significantly related to a positive HPV DNA result. Although it was surprising, it is a clear indicator of the living conditions of certain populations that can favour the spread of any infectious disease. The absence of an association between HPV positivity and other common risk factors described in other regions and populations (e.g. number of pregnancies, age at first intercourse, number of lifetime sexual partners, level of literacy) is not unexpected since the elevated HPV prevalence found in all age groups of this series tends to override the effect of such factors.

In relation to HPV-16 variants, the current epidemiological knowledge suggests that the non-European, and particularly the Asian-American variants (AA), are more closely related to CC development. In addition, more recent epidemiological evidence showed that among the European (E) branch, the non-prototypic variants (non-G1) could have a higher oncogenic potential[29-31]. We only found E and AA branches in the Pilagá women; the former predominated, accounting for 68.2% of the cases, but its prevalence was lower than in other indigenous groups of Brazil and Argentina[32-34]. Altogether, the proportion of non-prototypic, potentially oncogenic HPV-16 variants (i.e. non G1 E plus AA) was 54.5% in HPV-16-positive *Pilagá* women, providing an additional risk factor for CC development in this vulnerable community.

The prevalence estimates provided by this work clearly show that *Pilagá* women are highly exposed to or infected by HPV and therefore are at high risk of developing precancerous lesions and eventually CC at the population level. In this sense, it must be remembered that there is an estimated incidence of CC of 15.1/100 000 women/year (age standardized) in our study area. The Argentinean Health Ministry recently announced the implementation of vaccination strategies against diseases caused by HPV. This study provides valuable baseline knowledge about the prevalence and type distribution of HPV in Northern Argentina prior to the introduction of HPV vaccination.

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Procura descubrir la verdad por entre las promesas y dádivas del rico como entre los sollozos e importunidades del pobre.

Miguel de Cervantes (1547-1616)

Don Quijote de la Mancha. Segunda parte (1615). Capítulo XLII. De los consejos que dio Don Quijote a Sancho Panza antes que fuese a gobernar la ínsula, con otras cosas bien consideradas. Edición del IV Centenario. San Pablo, Brasil: Real Academia Española/Alfaguara, 2004, p 869