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Distribution of human papillomavirus (HPV) genotypes and bacterial vaginosis presence in cervical samples from Paraguayan indigenous

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SUMMARY

Objective: To determine the frequency of human papillomavirus (HPV) types and to assess bacterial vaginosis (BV) possible associations with cervical infections in indigenous Paraguayan women of the Department of Presidente Hayes.

Methods: This study included 181 sexually active women without cervical lesions. HPV typing was performed by polymerase chain reaction with primers PGMY 09/11 followed by reverse line hybridization. BV was diagnosed by the Nugent criteria using the results from a Gram stain smear.

Results: Sixteen percent of women were positive for at least one high risk HPV type (HR-HPV). The most frequent genotypes were HPV 16 (4.4%), followed by HPV 58 (3.3%), HPV 45 (3.3%), HPV 53 (2.8%) and HPV 11 (2.8%). A significant association between HR-HPV and BV was observed (p=0.01). In addition, women with BV had a higher frequency of *Chlamydia trachomatis* (p=0.0007), *Trichomonas vaginalis* (p=0.00009), *Mycoplasma hominis (p=0.001).*

Conclusions: A large variety of HPV genotypes was detected and showed a slightly different pattern from previous studies on urban women in Paraguay, with the predominance of HR-HPV. Furthermore, the information of co-infections involved in BV could be useful for the improvement of national prevention programs, as well as for laboratory surveillance of these genital infections.

1. Introduction

Cervical cancer is the fourth most common cancer in women, with 528,000 new cases and 266,000 deaths in 2012. In Paraguay, the standardized incidence and mortality rates are 34.2 and 15.7 per 100,000 women, respectively.[1]

It has been well established that persistent genital infection with high risk human papillomavirus (HR-HPV) is a necessary factor for developing cervical cancer.[2–4] Over 200 different HPV genotypes have been identified, of which about 40 infect the anogenital mucosa. They are classified into HR-HPV, low risk (LR-HPV), and others that are not yet classified. Fifteen HPV genotypes are classified as HR-HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59,

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68, 73, and 82), three types as probable HR-HPV (26, 53, and 66), and twelve as LR-HPV types (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108).[5,6] The prevalence of HPV infection and the distribution of HPV genotypes varies by geographic region and the severity of the cervical lesion.[7–11]

Because only HR-HPV persistent infection is not enough for cervical cancer development, it is considered that other factors would determine whether an HPV infection will resolve to normalcy or progress to high-grade lesions (HSILs) or cervical cancer; among the main risk factors are included: the prolonged use of hormonal contraceptives, high parity, smoking, co-infection with other sexually transmitted diseases, bacterial vaginosis (BV) and immune and nutritional deficiencies.[4,12] Their influence in the HPV infection acquisition and cervical cancer development is incompletely understood.

BV is characterized by a loss of *Lactobacillus* species and a concurrent overgrowth of anaerobic bacteria and the presence of potentially pathogenic bacteria, such as Bacteroides, Peptococcus, *Ureaplasma urealyticum*, and *Mycoplasma spp.*, which are most frequently detected in the vaginal tract.[12] However, the magnitude of association between co-infections involved in BV and HPV infection is varied in epidemiological studies and still remains controversial.[12,13]

Although some studies have investigated cervical infections in indigenous women in the Americas,[14,15] HPV and BV infections data in the native communities of Paraguay are limited.[14,15] Therefore, the aim of this study was to deepen knowledge of the epidemiology of cervical infections by determining the frequency of HPV genotypes, and assessing their BV possible associations with HPV infections in indigenous Paraguayan women of the Department of Presidente Hayes, Paraguay, which contains 23% of the native population of Paraguay.[16]

2. Materials and methods

2.1. Study design and sample collection

A cross-sectional study of 181 indigenous women from Presidente Hayes Department was performed. Recruitment is described in the work of Mendoza et al.[17] Briefly, the communities included were selected in conjunction with the local health authorities (Health Region XV), taking into account the availability of health workers who could facilitate access to women and perform sample collection. Indigenous communities were visited to explain the project and invite women to participate. The localities were found at a distance between 30 and 434 km from Asuncion, the capital of Paraguay. After selecting communities, the selection of women was conducted by sampling consecutive women who had sexual intercourse, were not pregnant, did not have mental illnesses, and did not have drug (vaginal ovules) or surgical (LEEP, cone hysterectomy, or other) treatments during the study period (October 2010 to November 2011). Of the 181 women included in the study, ethnicity was as follows: 40 were Maká, 23 were Nivaclé, 33 were Sanapaná, 51 were South Enxet, and 34 were Toba-Qom. This population constitutes 30% of the women population greater than or equal to 13 years.

Prior to sampling, all women signed a consent form and also completed a questionnaire about sociodemographics and gynaecological history. Subsequently, a gynaecologist took two cervical samples: one for cytological diagnosis and the other for the detection and typing of HPV. Data collection and analysis of results respected the confidentiality and anonymity of the patient. Each patient received the results of HPV detection and cytology. The local medical team conducted the monitoring and treatment of women.

2.2. Cytological diagnosis

Cervical brushing was performed by a trained gynaecologist. Results were reported according to the Bethesda 2001 System as negative for squamous intraepithelial lesions (NSIL), low grade squamous intraepithelial lesions (LSIL, which includes cervical intraepithelial neoplasm (CIN) I and/or HPV), high grade squamous intraepithelial lesions (HSIL, which includes CIN II and CIN III), or squamous cell carcinoma (SCC).[18]

2.3. Detection and typing of HPV

DNA extraction of cervical specimens was performed according to the methodology described by Mendoza et al.[11] in which the cell pellet was digested with proteinase K.

Detection and typing of HPV was performed according Estrade et al.[19] Briefly, generic PCR was performed using biotinylated PGMY09/11 primers, and the amplification product obtained was analysed by reverse line hybridization (RLB). Type-specific probes corresponding to 32 HPV types were used (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 6, 11, 26, 34, 40, 42, 43, 44, 53, 54, 55, 57, 69, 70, 73, 82, 83 [MM7], and 84 [MM8]). This approach allows the evaluation of the quality of the DNA sample by co-amplification of a 230 bp HLA fragment. To detect positive samples for other HPV types not included in the RLB probes, the amplification products obtained using PGMY09/11 primers of all RLB negative samples were subjected to electrophoresis on polyacrylamide gels to determine whether an HPV-specific band of 450 bp was present. In particular, in the case of HPV studies, the criteria adopted for considered a positive woman for HPV was a positive result in PGMY09/11. The criteria for the follow-up was women who tested positive for HPV but had negative cytology result would be followed with a control in a year; women who tested positive for both HPV and cytology, would be referred to colposcopy.

For the classification of HPV genotypes into HR-HPV, probable HR-HPV, and LR-HPV, the Muñoz et al. classification was used.[6]

BV was diagnosed by the Nugent criteria using the results from a Gram stain smear of the vaginal swab.[20] A score of 0 to 3 was considered normal, 4 to 6 was classified as abnormal vaginal flora (or intermediate), and 7 to 10 was defined as BV.

Detections of others infections was performed by either Gram staining and/or cultura (for *Candida* sp., *Trichomonas vaginalis, Gardnerella vaginalis*), serological methods (for *syphilis) and* molecular methods (for *Chlamydia trachomatis, Mycoplasma hominis y Ureaplasma urealyticum)* as is detailed in Mendoza et al.[17]

2.4. Statistical Analysis

Data analysis was performed using Epi Info version 3.5.3 (Centers for Disease Control and Prevention, Atlanta, GO, USA). To determine the frequency of HPV types in the population, single and multiple HPV infections were considered either separately or combined. HPV frequency was estimated within broad age groups (<30, 30–39, 40–49 and >50 years).

To estimate the possible association between the variables analysed, the Chi-square test was used, and $p \leq 0.05$ was considered statistically significant.

3. Results

A total of 42/181 women (23%; 95%CI: 17%–30%) were positive for HPV, including 24 women (13%; 95%CI: 8%–19%) with single infections, 17 with multiple infections (9%; 95%CI: 5%–14%), and one woman with HPV infection of an undetermined type (0.6%; 95%CI: 0%–3%). Women with HPV infections had an earlier age of

Table 1
HPV frequency according socio-demographic and sexual characteristics of 181 indigenous women

Characteristics	Total (n: 181)	HPV positive women (n=42)	HPV negative women (n=139)	P
Age (years)				
Median (Interquartile range)	30 (23-41)	27 (20-40)	30 (24-42)	
<27 (n %)	64	19 (30)	45 (70)	p=0.13
≥27 (n %)	117	23 (20)	94 (80)	
Education (n %)				
Illiterate / Elementary school	168	40 (24)	128 (76)	p=0.49
High school	13	2 (15)	11 (85)	
Age at 1st sexual intercourse(years)				
Median (Interquartile range)	16 (13-19)	16 (14-18)	17(14-20)	
≤16 (n %)	105	31 (30)	74 (70)	p=0.018
16 (n %)	76	11 (14)	65 (86)	
n sexual partners				
Median (Interquartile range)	1 (1-2)	2 (1-3)	1 (1-2)	
2 (n %)	28	11 (39)	17 (61)	p=0.028
≤2 (n %)	153	31 (20)	122 (80)	
Pregnancies				
Yes (n %)	166	39 (23)	127 (77)	p=0.52
No (n %)	15	3 (20)	12 (80)	
n pregnancies (n=166)				
Median (Interquartile range)	3 (1-5)	3 (2-5)	3 (1-5)	
>3 (n %)	80	18 (22)	62 (78)	p=0.77
≤3 (n %)	86	21 (24)	65 (76)	
Oral contraceptive use				
Yes (n %)	107	23 (21)	84 (79)	p=0.51
No (n %)	74	19 (26)	55 (74)	
Smoking				
Yes (n %)	39	11 (28)	28 (72)	p=0.40
No (n %)	142	31 (22)	111 (78)	
Previous Cytology				
No (n%)	130	28 (22)	102 (78)	p=0.40
Yes (n%)	51	14 (27)	37 (73)	

HPV, human papillomavirus; IC, Confidence Interval.

sexual intercourse (16 years old or lower, p = 0.018) and a higher number or sexual partners (two or more, p = 0.028), Table 1.

Sixteen percent of women (29/181) were positive for at least one HR-HPV type, 5% (9/181) for at least one type of probable HR-HPV, and 11% (19/181) for at least one type of LR-HPV. The most common type was HPV 16 (4.4%), followed by HPV 58 (3.3%), HPV 45 (3.3%), HPV 53 (2.8%), and HPV 11 (2.8%), Table 2. All women had a NSIL cytology result, and 14% (95%CI: 9%–20%) had inflammatory cytology.

Table 2
Frequency of HPV genotypes among indigenous women of the Department of Presidente Hayes

	Total (n=181)[a]	Women with single infection	Women with multiple infection[b]	Undetermined type
HPV	42	24	17	1
HR-HPV	29 (16)	13	16	
16	8 (4.4)	4	4	
18	1 (0.6)	0	1	
31	4 (2.2)	0	4	
35	1 (0.6)	1	0	
39	2 (1.1)	0	2	
45	6 (3.3)	2	4	
51	1 (0.6)	0	1	
52	3 (1.7)	1	2	
56	1 (0.6)	0	1	
58	6 (3.3)	3	3	
59	1 (0.6)	1	0	
68	2 (1.1)	0	2	
73	3 (1.7)	1	2	
82	3 (1.7)	0	3	
Probable HR-HPV	7 (3.9)	5	2	
53	5 (2.8)	3	2	
66	3 (1.6)	2	1	
LR-HPV	19 (10.5)	5	14	
6	2 (1.1)	0	2	
11	5 (2.8)	2	3	
44	3 (1.7)	1	2	
54	2 (1.1)	0	2	
70	1 (0.6)	0	1	
83	4 (2.2)	0	4	
84	4 (2.2)	2	2	

HPV, human papillomavirus; HR-HPV, high risk oncogenic HPV; LR-HPV, low risk oncogenic HPV.
[a] Frequency of HPV genotypes detected in women with single and multiple infections.
[b] Frequency of HPV genotypes detected in women with multiple indigenous infection.

Table 3
Frequency of Nugent score according to HPV and HR-HPV results ofindigenous women

Nugent score	HPV Positive n (%)	HPV Negative n (%)	P	HR-HPV Positive n (%)	HR-HPV Negative n (%)	P
0–3 (normal), n: 92	13 (31)	79 (57)	0.0033	8 (27.5)	84 (55.3)	0.006
4–6 (intermediate), n:32	11 (26)	21 (15)	0.10	6 (20.7)	26 (17.1)	0.64
7–10 (BV), n: 57	18 (43)	39 (28)	0.07	15 (51.7)	42 (27.6)	0.01
Total	42 (100)	139 (100)		29 (100)	152 (100)	

HPV, human papillomavirus; HR-HPV, high risk oncogenic HPV; BV, bacterial vaginosis.

Table 4
Frequency of genital infections according to bacterial vaginosis results of indigenous women

Variables	BV (n=57) n (%)	No BV (n=124) n (%)	P
Gardnerella vaginalis	55 (96.5)	28 (22.6)	0.00001
Chlamydia trachomatis	12 (21.0)	6 (4.8)	0.0007
Trichomonas vaginalis	13 (22.8)	5 (4.0)	0.00009
Syphilis (VDRL & IgG*)	10 (17.5)	11 (8.9)	0.09
Candida sp	2 (3.5)	11 (8.9)	0.32
Mycoplasma hominis	27 (47.4)	29 (23.4)	0.001
Ureaplasma urealyticum	11 (19.3)	26 (21.0)	0.796

BV, bacterial vaginosis.

Fifty seven out of 181 women (31.5%; 95% CI 24.8% to 38.8%) were positive for BV, 32 (17.7%; 95% CI 12.4% to 24.0%) had an abnormal vaginal flora (or intermediate) and 92 (50.8%; 95% CI 43.3% to 58.3%) had a normal vaginal flora. Women with vaginal normal flora had lower frequency of HPV infection. In addition, a significant association between HR-HPV and BV was observed (Table 3).

No significant associations between sociodemographic, sexual characteristics and the present of BV were seen (data not shown). However, women with BV had a significant higher frequency of *Chlamydia trachomatis*, *Trichomonas vaginalis* and *Mycoplasma hominis* infections (Table 4).

4. Discussion

Few studies can be found in the literature regarding epidemiological surveys of sexual transmitted infections on American Indian populations, being South American tribes particularly ignored. This report provides the first data on the frequency of HPV genotypes, BV and the evaluation of their associations with possible risk factors among indigenous women in Paraguay.

The any-type HPV prevalence in these women (23.2%) was comparable to those found by Mendoza et al. (20.8%) in Paraguayan Caucasian women with negative cytology, but lower than the frequency detected in previous studies performed with Guarani and Quechua Argentine indigenous (64% and 52%, respectively), Brazilian Amazon (42.85%) and Venezuelan Amazon (57%) tribes; however, the HPV prevalence detected in this study was higher than the 13.2% observed in a meta-analysis including South American urban women with normal cytology, according it was previously discussed in Mendoza et al.[17]

In the present study, HPV 16 was the most common genotype found in single and multiple infections, which is similar to previous reports.[7–11,21] Cohort studies have found that women with persistent HPV 16 infection are at a higher risk of developing HSIL than women infected with other HR-HPV types.[22–24] The HPV 16 prevalence that we found was 4.4% (95%CI: 1.9%–8.5%), which is higher than the global prevalence in women with normal cytology (3.2%), but is similar to local reports (4.3% and 5.5%).[7,11,21] However, the HPV 16 prevalence that we report is much lower than those obtained in indigenous populations from Argentina (52% and 64% in Quechua and Guarani women, respectively).[14,15] These studies also found more risk factors for acquiring HPV infection than in the present study, such as early sexual exposure, multiple sexual partners, and a higher number of pregnancies per woman. The discrepancies may be due to differences in the applied genotyping strategies, but also to own socio-cultural and environmental conditions of each community with semi-closed characteristics.

After HPV 16, HPV 45 and 58 were the most commonly detected genotypes. This type-specific prevalence was slightly different from the result reported in previously studies conducted in Asunción in NSIL Paraguayan women. Mendoza et al. observed a frequency of HPV 31/58 of 2.4% each, Rolon et al. observed a frequency of HPV 31/35/58 of 2.2% each, and both studies observed a lower frequency of HPV 45 than we report (1.4% by Mendoza et al. and 1.1% by Rolon et al.)[11,21]

It is important to underline the higher HPV 45 prevalence reported in this study, because it is not typically between the most common genotype observed in women with normal cytology.[25] However, the detection of HPV 45 is very important because of its presence in cervical adenocarcinomas; in the study of Sanjose et al.[9] it was detected together with HPV 16 and 18 in almost 94% of cases of invasive cervical cancer. In addition, Sanjose et al.[9] and Muñoz et al.[26] observed a faster development of cervical cancer in women positive for HPV 16, 18, and 45 compared with women with cervical cancer with other HR-HPV genotypes. Therefore, one might suggest more careful monitoring in young women with persistent infection with HPV 45 (as has been proposed for HPV 16 and 18) to prevent the development of lesions and cancer. Beyond differences in type-specific frequencies observed in different reports, these data reconfirm the high prevalence of HR-HPV, even in non-pathological cytology.

In this study, a higher frequency of multiple infections was detected (9.4%) than reported by Mendoza et al.[11] in non-indigenous women with NSIL (4.3%). There is controversy regarding whether multiple infections with HPV are related to a higher risk of development of cervical lesions.[27,28] However, laser-capture microscopy (LCM)-polymerase chain reaction (PCR) confirms that only 9% of histologic CIN2/3 is associated with multiple HR-HPV types and each lesion was associated with a single HR-HPV infection.[29]

A significant association was observed between HPV infection and first sexual intercourse at 16 years of age or younger ($p = 0.018$), as well as a greater number of sexual partners (more than two) ($p = 0.028$). These results are in agreement with several studies.[30–32] Regarding the number of sexual couples, some studies report a higher probability of acquiring HPV infection in women with many sexual partners and this fact is one of the most important risk factors for acquiring an HPV infection.[33,34]

Determining the viral type distribution in native communities is of special epidemiological interest, because it constitutes a potential infective reservoir available for viral spreading into new populations once contacts begin to be established. Another point to take into account is that these groups often migrate among settlements located in northeastern Argentina and southern

Paraguay. This behaviour is a consequence of the nomad character of the Guarani tribes and its agricultural practices. During this transit, contacts with white population are beginning to be established, confronting people to different sanitary conditions and hygiene standards, making transmission of infectious diseases more likely.

In this study was observed a high frequency of BV (31.5%). Studies have now conclusively shown that lactobacilli-dominated are associated with a healthy vaginal micro-environment and that BV is best described as a polybacterial dysbiosis: the *Lactobacillus sp* load decreases, and both the diversity and bacterial load of other (facultative) anaerobic bacteria increase.[35,36] Furthermore, in agreement with others studies, we observed an association between BV and HR-HPV, *Trichomonas vaginalis*, *Chlamydia trachomatis*.[37–39] These results suggest that in presence of BV it is necessary to implement a joint screening of other genital infections of clinical importance.

For the majority of women (72%), this was the first time they had been screened by cervical cytology. This adds value to the present study by allowing women with poor access to the health system the chance to receive a gynaecological test and health care information, which is crucial in the cervical cancer prevention.

5. Conclusion

This work confirms the high prevalence of HPV infection in cross-sectional measurements among aboriginal women from Paraguay with normal cytological findings, and it further indicates that the vast majority of detected HPV infections include high-risk types. This result could explain in part the high cervical cancer incidence in this country. The study had the additional social value of permitting gynaecological screening access and health care education to many indigenous women of Paraguay. In addition, there was observed an association between BV and HR-HPV, *Chlamydia trachomatis*, *Trichomonas vaginalis, Mycoplasma hominis*. This information could be used by national health authorities for the improvement of prevention programs, as well as for laboratory surveillance of these genital infections.

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Ethical approval: The study protocol (P11/2010) was approved by the Ethics Committee of the Institute for Research in Health Sciences of the National University of Asunción. All women signed a consent form written in Spanish prior to the sampling of biological material and the application of a questionnaire to collect data related to socio-demographic and sexual characteristics of indigenous women. In the case of indigenous women who did not speak Spanish, informed consent was translated to her language by a community member. Furthermore, in the case of women under 18 years old, informed consent was signed by the parent or guardian.

Competing interests: The authors declare that they have no competing interests.

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