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HPV genotyping in anogenital abnormal samples of Ecuadorian women

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Abstract. *Background:* Limited data are available describing human papilloma virus (HPV) genotype distributions in gynecological lesions in Ecuador. To predict how HPV vaccination and HPV-based screening will influence cervical cancer prevention it needs such studies.
Methods: We analyzed 124 samples from women, adults between 18 to 55 years old, Mestizas (Hispanics), were born and living in Quito, Ecuador. They showed an atypical sample in PapTest or a histological abnormal evaluation. We used the kit PVH Fast® 2.0 with conventional PCR to study cervical and vulvar swabs prior colposcopy and/or cytology.
Results and conclusions: We found 23 different genotypes. 84/104 cases were positive for HPV (67.7%); 32/124 cases were negative (25.8%); and, in 8/124 cases (6.5%) we were unable to ascertain the existence or lack of HPV. The most common viral genotype was 6 (8.8%) followed by the 66 (4.8%) and 16, 31, 44 types (2.4% each one). Types 11, 34, 35, 54, 59, 62 and 67 showed a frequency equivalent to 1.6% each one. The remaining types showed a 0.8% frequency. Most common high-risk genotypes were 16 and 31, low-risk 6 and 41 and, the third most common group HPV type detected in this cohort was HPV 66. HPV 6 showed the highest prevalence. HPV 66 was associated with atypical cytology and was found in women with borderline cytology, low-grade lesions and high-grade lesions, but was most frequent in the threshold group. We examine a case of coexistence of 2 genotypes together 51 and 58. We found very low prevalence of HPV18 alone or HPV16/18. In 25 samples, 20.16% (25/124) we found HPV presence but we were unable to identify the genotype. We need more studies with a broad sampling to complete our geographic pattern of HPV distribution.

Keywords: HPV, PCR, Quito, Ecuador, genotyping, cervical cancer, risk factor, screening, prevalence

Abbreviations

HPV	human papilloma virus
ICC	invasive cervical cancer
CIN	cervical intraepithelial neoplasia
PCR	polymerase chain reaction
CIS	Carcinoma in situ
SCC	Squamous Cervical Carcinoma
LIE	lesion intraepithelial
H-SIL	high grade squamous intraepithelial lesions
L-SIL	low grade squamous intraepithelial lesions

1. Introduction

Disparities in cervical cancer incidence and anogenital lesions rates exist among women of different origins. It have not been enough studies to establish the existing prevalence of Human Papillomavirus (HPV) infection in our population [1,2]. Mostly collected samples arising from private medical offices, and two of them studied immigrant women on specific sexual groups outside of Ecuador [3,4], while others researchers focused HPV infection in different pathologies, always using Ecuadorian samples [5,6]. Some authors pointed out the threat of certain HPV types being more widespread in women in Latin-American Countries than elsewhere [7–9]. As seen, there are not

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extensive population studies in women from Ecuador about HPV and diseases, especially when there is not a discernible link between HPV and ethnicity. Actually, three major ethnic groups reside in Ecuador: Mestizos, Amerindian Natives, and Afro-ecuadorians Blacks [10, 11].

HPV is a double stranded DNA virus assigned to Group I, family Papillomaviridae. It is the leading source of cervical intraepithelial neoplasia (CIN) and invasive carcinomas of the uterine cervix [12]. If untreat the persistent cervical infection with HPV the cervical dysplasia related with this infection, could potentially progress to invasive cervical cancer (ICC). It estimates that Invasive Cervical Cancer (ICC) is the most frequent malignancy affecting women in developing countries. It affects roughly 500,000 women each year, rising almost an 80% [13]. High-risk genotypes 16 and 18 cause persistent infection and produce 70% of all cervical cancers [14]. Types 6 and 11 do not contribute to the incidence of high-grade dysplasia (precancerous lesions) or cervical cancer but do cause laryngeal papilloma and most genital warts [15]. HPV is highly transmissible, with peak incidence soon after the onset of reproductive activity [16]. Genital HPV infection is typically transmitted by genital skin-to-skin contact, usually although not necessarily during sexual intercourse [17]. HPV infection can occur at any age [18]. The amount of cervical cancer with high and low-grade squamous intraepithelial lesions (HSIL and LSIL) is due to different HPV genotypes; but even persist some gaps in Central Asia, Africa and Eastern Europe [19], with the predictable exception of Europe. The same eight HPV genotypes were the most numerous in each region [20]. The relative observed prevalence of HPV genotypes 31, 33, 35, 45, 52 and 58 differed by region [21]. After HPV-16/18, the six most common HPV types are the same in all world regions, namely 31, 33, 35, 45, 52 and 58; these account for an additional 20% of cervical cancers worldwide [22]. It estimates that overall HPV prevalence in women with conventional cervical cytology is 10.4% (95% CI 10.2–10.7). In all world regions, HPV prevalence was highest in women younger than 35 years of age, decreasing in women of older age. In Africa, the Americas, and Europe, it observes a second peak of HPV prevalence in women aged 45 years or older. On the basis of these estimates, around 291 million women worldwide are carriers of HPV DNA, of whom 32% are infected with HPV16 or HPV18, or both [23]. The aim of this work is established the most frequent HPV types in Ecuadorian women with abnormal anogenital lesions.

Box 1

Source	N =	Fq
From uterine cervix		
Brushing of the cervix*	73	0.584
Biopsy of endocervix	20	0.160
Biopsy of exocervix	10	0.080
Brushing of endocervical channel*	1	0.008
Exocervix (paraffin block)	1	0.008
Subtotal	105	0.84
From genital inferior tract		
Anal and genital warts	8	0.064
Biopsy of vulva	6	0.048
Biopsy of vagina	2	0.016
Biopsy of hymeneal edge	2	0.016
Vulvar warts	1	0.008
Subtotal	19	0.16
Overall	124	1

*Fresh pap-smear samples. Sensibility is different depending of the samples. Fq = frequency.

2. Material and methods

2.1. Population

124 samples were analyzed in this study. Inclusion criteria were: women, adults between 18 to 55 years, Mestizas (Hispanics), born and living in Quito. They showed an atypical sample in PapTest or a histological abnormal evaluation. During a enroll period of 1 year we collected the samples. Samples came from patients of the Gynecological outpatient facility of the Axxis Medical Center and the Metropolitan Hospital in Quito. Pathology studies were made by consultants in independent practice. This study was exempt from Institutional Review Board approval due to internal policies.

2.2. Samples

Two kinds of samples were analyzed, the first type arises from a cervical cytobrush (fresh samples), and the following group arises from biopsy with colposcopy taken by a Gynecologist and sent them to the laboratory. It took the precaution of use the samples that they had not been placed Acetic Acid or Iodine solutions previously. During the next 24 hours of collection, the fresh samples were processed. The cervical cytobrush (fresh) samples were transported in essay tubes with no additives or preservatives media. The cervical cytobrush (fresh samples) were not taken at the identical time as samples for PapTest or histological analysis. Box 1 shows the source of the samples according with the origin, from the uterine cervix or genital inferior tract

Table 1
Comparison with prior studies

Ref	Samples	N=	HPV+	HPV-	UN	HR	LR
Paez et al., 1996	Paraffin-embedded tissue	161	80 (50%)	81 (50%)	17 (21%)	16,18	6, 11
M. Tornesello et al., 2008	Paraffin-embedded tissue	71	31 (43%)	40 (56%)	1 (3.2%)	16, 53, 56, 58	81
J. Del Amo et al., 2005	Cervical swabs	158	66 (42%)	92 (58%)	ND	ND	ND
C. González et al., 2006	Frozen tissues	26	6 (23%)	20 (77%)	ND	16, 18, 31, 33, 58, 66	ND
Our study	Fresh pap-smear samples, biopsy tissues and Paraffin-embedded tissue	105	85 (81%)	20 (19%)	8 (6%)	16, 31, 39, 44, 61, 66	6, 11

HR = High risk genotypes founded, LR= Low risk genotype founded, ND = No data available, Samples = Type of samples analyzed, UI = undetermined genotypes.

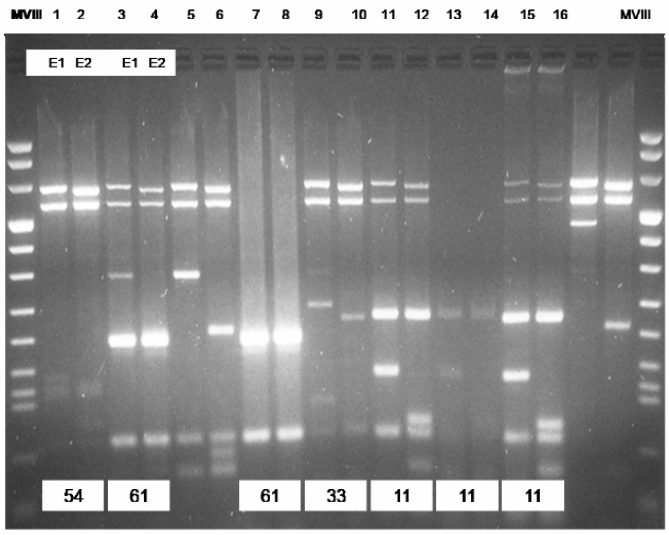

Fig. 1. It shows restriction patterns in a high resolution 4% polyacrilamide gel. E1 = Restriction enzyme 1, E2 = restriction enzyme 2. Line 3, 4, 7 and 8 show amplification of genotype 61; line 1 and 2 genotype 54; line 9 and 10 genotype 33; lines 11, 12, 15, 16 genotype 11. HPV extraction, amplification and detection were made according to manufacturer recommendations (PVH Fast® 2.0, Genomica).

2.3. Procedure

We used the kit PVH Fast® 2.0 (Genomica SA, Madrid, Spain) with a conventional PCR. Genotyping method is limited to find 52 different HPV types. We collected samples by duplicate and we stored the specimens at 4°C, for 3 days before detection, at maximum. We carried out HPV extraction, amplification and detection according to manufacturer recommendations (PVH Fast® 2.0, Genomica). We amplified a 450 bp fragment of L1 ORF region using the MY09/11 consensus primers, in a thermocycler model Genius © (Techne). We co-amplified an internal control and a endogenous human CFTR gene markers. We used a high resolution 4% polyacrilamide gel and the electrophoresis in a Gibco BRL Sequencing System, model SA, (Gibco©, USA) from 1,200 V to 2,200V at the end, for detection and typing of amplicons (RFLPs). See Fig. 1.

3. Results

The Table 1 shows a correlation between our results and comparable studies made with samples of Ecuadorian women. Tables 2 and 3 show a correspondence between the pathological findings and the HPV genotyping. Finally, Table 4 presents the most common genotypes founded in this study. We found 23 different genotypes. 84/104 cases were positive for HPV (67.7%); 32/124 cases were negative (25.8%); and, in 8/124 cases (6.5%) we were unable to ascertain the existence or lack of HPV. The most common viral genotype was 6 (8.8%) followed by the, 66 (4.8%) and 16, 31, 44 types (2.4% each one). Types 11, 34, 35, 54, 59, 62 and 67 showed a frequency equivalent to 1.6% each one. The remaining types showed a 0.8% frequency. Most common high-risk genotype were 16 and 31, low-risk 6 and 41 and interestingly, the third most com-

Table 2
Correlation between histological findings and molecular HPV test in *Uterine Cervix samples*

Nomenclature	HPV+ ($n = 85$)	HPV- ($n = 20$)	N = 105
HPV	8	1	9 (0.346)
Mild Dysplasia + HPV	4	2	6 (0.230)
Class II, inflammmatory	3	2	5 (0.192)
Class III A	2	0	2 (0.076)
Severe Dysplasia + HPV	1	0	1 (0.038)
Class II B	1	0	1 (0.038)
Moderate Dysplasia + HPV	0	1	1 (0.038)
Intraepithelial Lesion	0	1	1 (0.038)
HPV Recidivation + CIN	1	0	1 (0.038)
Normal prior test	65	13	98 (0.784)
	85	20	105
	80.9%	19.1%	

Nomenclature is used for the *Histology:* CIN = Cervical Intra-epithelial neoplasias, CIS = Carcinoma in situ, and SCC = Squamous Cervical Carcinoma, IL = intra-epithelial lesion. Recidivation of HPV = relapse of the disease or symptom.

Table 3
Correlation between histological finding and presence of HPV, samples arising of inferior genital tract

Histology description	HPV+	N = 19
Vaginal and hymeneal lesions		
Inflammatory + myld dysplasia	4	0.21
Vulvar lesions		
Vulvar warts	1	0.05
Lichen sclerosus	4	0.21
Squamous hiperplasia	2	0.11
Anal and perineal lesions		
Anogenital warts (condylomata acuminata)	7	0.37
Inflammatory + myld dysplasia	1	0.05
Overall	19	1

ISSVD Classification for *Epithelial Vulvar Disease*:
1. Nonneoplastic epithelial disorders of vulva and mucosa (Lichen sclerosus, Squamous hyperplasia, Other dermatoses);
2. Mixed neoplastic and nonneoplastic disorders;
3. Squamous vulvar intraepithelial neoplasia, VIN;
4. Non-squamous intraepithelial neoplasia.

mon group HPV type detected in this cohort was HPV 66, which has not been reported before in Ecuadorian HPV genotyping studies. The types of low risk (L-SIL) grouped accounted for 13.6% of the overall and high-risk (H-SIL) 11.2%. We report a high percentage of unspecific genotypes in 25 samples. We also analyzed biopsies taken at distinct sites of inferior genital tract like vulva, vagina and the hymeneal edge. We analyzed only one case set previously in paraffin. 34.6% of cases had a positive previous Pap test suggesting HPV; from them 23% reported mild dysplasia and HPV; 19.2% had prior reports of Pap Class II nonspecific inflammatory, 7.6% had a previous report Class III type A.

Table 4
HPV type founded by frequency and rank

Type	N=	Fx	Rank	Type	N=	Fx
6	**11**	**0.088**	**1**	**6**	11	0.088
11	2	0.016	**2**	**66**	6	0.048
13	1	0.008	**3**	**16**	3	0.024
16	**3**	**0.024**	**4**	**31**	3	0.024
18	1	0.008	**5**	**39**	3	0.024
31	**3**	**0.024**	**6**	**44**	3	0.024
34	2	0.016	**7**	**61**	3	0.024
35	2	0.016	8	11	2	0.016
39	**3**	**0.024**	9	34	2	0.016
40	1	0.008	10	35	2	0.016
42	1	0.008	11	54	2	0.016
44	**3**	**0.024**	12	59	2	0.016
51	1	0.008	13	62	2	0.016
52	1	0.008	14	67	2	0.016
53	1	0.008	15	13	1	0.008
54	2	0.016	16	18	1	0.008
58	1	0.008	17	40	1	0.008
59	2	0.016	18	42	1	0.008
61	**3**	**0.024**	19	51	1	0.008
62	2	0.016	20	52	1	0.008
64	1	0.008	21	53	1	0.008
66	**6**	**0.048**	22	58	1	0.008
67	2	0.016	23	64	1	0.008
Unspecific	25	0.200				
Total	125	1				
Types	N=	Frequency				
LR types[1]	17	0.136				
HR types[2]	14	0.112				
Unspecific	25	0.200				
Others	69	0.552				

LR = Low risk genotypes: 6, 11, 42, 43, 44.
HR = High risk genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68.

4. Discussion

Detection of specific HPV genotypes may be useful for differentiating between those women who are carcinogenic HPV-positive at lower and higher risk for cervical pre-cancer and cancer. Considerable evidence already exists to validate this information. Based on extensive case series, meta-analyses, and laboratory data of mechanistic studies, twelve HPV genotypes (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) were deemed carcinogenic; and HPV68 was considered probably carcinogenic too [29].

Detection of persistent carcinogenic HPV is strongly associated with cervical pre-cancer and cancer and predicts its development, and might be used to track the outcomes of HPV infections. Each genotype of HPV has differing carcinogenic risks linked to evolutionary species and acts as an independent infection. HPV genotypes act independently such that cancers with multiple HPV genotypes detected are proportionally attributable to the HPV genotypes according to their

overall prevalence; and HPV causes all cervical cancer, and false negative results are independent of HPV genotype. It exist an overlapping between HPV genotypes due to multi- HPV genotype infections (∼25% of HPV positives).

4.1. HPV 6

The prevalence of HPV6 in this study was 8.8%, highest prevalence and the most common low-risk type founded in our study. Genital warts (GW) have a relationship with this genotype, which are widespread, and increasing in young people. HPV 6 and 11 are reliable for up to 90% of anogenital warts and virtually all cases of recurring respiratory papillomatosis [24,25].

The DNA of HPV-6 does not incorporate into cellular DNA, in high-grade squamous intraepithelial lesion (HSIL) of the uterine cervix. GW also known like Condyloma acuminate (CA) is sexually transmitted and appears with a distinctively papillary pattern. It can be diagnosed by attentive observation and is among the most prevalent sexually transmitted diseases. Although it is understood that low-risk HPV genotypes 6 and 11 are associated with CA, there have only been a few, small, published studies reporting the genotype-specific prevalence of HPV.

4.2. HPV 66

The following most frequent type detected in this cohort was HPV 66, which has not been reported previously in Ecuadorian HPV genotyping studies. HPV 66 was associated with atypical cytology and was found in women with borderline cytology, low-grade lesions and high-grade lesions, but was most frequent in the threshold group. In the current study, we identified HPV 66 in women with abnormal cytology. Recently, studies made in France [26] and Guatemala [27] found the highest prevalence of HPV 66 in LSIL lesions. HPV-16 was primarily associated with the probable high-risk genotypes: HPV-53 and HPV-66.

In 2003 [28], the International Agency for Research on Cancer (IARC) proposed an epidemiological classification of the HPVs regarding their oncogenic potential: 15 HPV types were classified as high-risk types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), 3 were classified as probable high-risk types (types 26, 53, and 66), and 12 were classified as low-risk types (types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108).

In 2009 [29], IARC downgraded HPV66 from the category of probably carcinogenic (Group 2A), as judged in 2005, to possibly carcinogenic (Group 2B), which also includes HPV genotypes HPV26, HPV53, HPV67, HPV70, HPV73, and HPV82. Given the high prevalence of some under diagnosed HPV types in the population (principally HPV53, HPV66, HPV84, and HPV87) and their continual association with cytological abnormalities, techniques capable of detecting and typing them would prove particularly useful.

HPV 66 is classified taxonomically in alpha HPV genus-species 6, together with HPV-30, HPV-53, HPV-56, HPV66, HPV67, HPV69, HPV70, and HPV85 are related from phylogenetic point of view [30]. Only a multicentric analysis, based on a particularly considerable amount of patients, could clarify this aspect. Worldwide, the prevalence of HPV66 estimated to be 0.4% (95%CI = 0.3–0.4%) although, there are some local variations in its prevalence [23]. Whether these HPV66-related CIN2/3 cases are clinically important, i.e. have invasive potential, or not remains unclear.

4.3. HPV 16

HPV genotype 16 alone was the most common high-risk type detected in the existing study (2.4%). This class is linked to more than 50–55% of all cervical cancers [31]; thus, the prevalence of HPV 16 is of particular interest. It is consistently the most common HPV type contributing to invasive cervical cancer cases strongly suggesting that this viral type has a biological advantage for transmission, persistency and transformation.

While the majority of infections are asymptomatic or self-limited, acquisition of specified types of HPV can result in clinically important disease. Most prominent among the oncogenic types (i.e., high-risk types) of HPV are types 16 and 18, responsible together for approximately 70% of all cervical cancer [32] as well as a lesser proportion of cancer of the vagina, vulva, penis, anus, mouth and pharynx [33].

4.4. HPV 18

We found very low prevalence of HPV18 alone or HPV16/18. This is similar with preceding studies, performed in various other Latino countries, which detected relative low frequency HPV-18 genotypes in pre-neoplastic lesions as well as neoplastic lesions. Around 291 million women worldwide are carriers of HPV DNA, of whom 32% are infected with HPV16 or HPV18, or both [34]. Worldwide comparison of HPV-

16 and 18 isolates revealed a distribution of different genomes that correlated with the geographic origin and the ethnicity of the infected cohort and led to the concept of unique African, European, Asian, and Native American HPV-16 and 18 variants.

The HPV genome includes several open reading frames that encode proteins involved in viral DNA replication (E1 and E2), viral gene expression regulation (E2), virus assembly (E4) and the immortalisation and transformation of infected epithelial cells (E5, E6 and E7; high-risk HPV only). The open reading frames L1 and L2 encode the two capsid proteins. HPV target stem cells of the squamous epithelium. Viral DNA integration occurs with high-risk types and leads to the over expression of two viral oncoproteins, E6 and E7. These proteins, in combination with E5, promote the immortalisation and transformation of infected cells.

4.5. Multiple infections

We examine a case of coexistence of 2 genotypes together 51 and 58. HPV16/18 can coexist, too. The detection of multiple HPV infections is not a unique or unusual event; it has been ell described that almost 25–30% of samples harbour more than one HPV genotype [35] of more than 200 human HPV types presumed to exist. Many HPV infections are sustained by multiple viral genotypes, whose effect on the risk of CIN is unknown.

One analysis [36] found that HPV prevalence was 99.4% with a 72.1% rate of co-infection. The risk of co-infection was higher for types 6, 11, 16, 18, 31, 33, 51, 52, 56. Co-infection by types 31-35-56, 16-51-52, 16-18 and 51-52 was more numerous than expected. Interactions between viral species and HPV 16-18 were maintained among CIN1, whereas interactions of 16-51-52 and 31-51-56 were important only in CIN>/=2. Interactions between strain and types were lost among women younger than 32 years [37]. Significant clustering of HPV types and species occurs among women with CIN. This has implications for the assessment of the oncogenic potential and the prevention of HPV infections.

4.6. Unspecified genotypes

In 25 samples, 20.16% (25/124) we found HPV presence but we were unable to identify the genotype. Most likely the used method was limited to detect all the types, even when it is capable of identify 52 types of the overall 200 described. Of them, at least 40 genotypes of HPV affect to anogenital region. It attempt to decide whether these borderline carcinogenic HPV genotypes are cancer causing or not. Integrative epidemiological and further laboratory approaches will be needed for clarification. New investigative tools, such as microdissection of the lesion and HPV genotyping or *in situs* methods if they ever prove to be sufficiently sensitive and genotype-specific detection of HPV E6/E7 transcripts or protein may help to assign causal HPV genotypes.

4.7. Screening recommendations

The American Cancer Society recommends that all women should start having a Pap test about 3 years after they begin having sex (vaginal intercourse), but no later than age 21. The test should be done every year if the conventional Pap test is used, or every 2 years if the newer liquid-based Pap test is used. For women over 30 they suggest to have one of the Pap tests every 3 years plus the HPV DNA test. Population-based studies of HPV genotype prevalence are needed to predict how these approaches might influence cervical cancer prevention. The findings of this paper may not be generalize to disparate populations.

Prevention programs that will involve the application of the more traditional screen, colposcopic evaluation of screen positives, and treatment of women with precancer will need sufficient numbers of highly-trained colposcopists to handle the clinical volume of screen positives, which could approach 10–15% of the population in multiple locations. However, the adoption of any cervical cancer screening and prevention program will depend on its social and cultural acceptance as well as handy resources and the political willpower to implement it. We need more studies with a broad sampling to complete our geographic pattern of HPV distribution.

4.8. Competing interests

The authors declare that they have no competing interests.

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