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Relevant Frequency of Multiple Infections with High- and Low-Risk HPV Genotypes among Mexican Women Attending a Tertiary Care Hospital

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Abstract

Aims: To assess the frequency and the main HPV genotypes circulating among a group of women attending at a third level Hospital in Mexico City. Methods: A cross-sectional and descriptive study was performed in a group of 143 female outpatients of the Gynecology and Obstetrics Service at the National Institute of Perinatology of Mexico. Cervical swabs were taken from participants and subjected to simultaneous detection/genotyping of HPV by Linear Array Genotyping Test (Roche Molecular Systems). Mann-Whitney U, median and/or Square Chi tests were used to compare socio-demographical features between HPV-infected and uninfected women. Results: A total of 66 women (46.2%) had HPV infection. Overall, 112 genotypes were detected either as single infections (45.5%) or multiple genotype infections (54.5%). The cumulated frequency of multiple infections with high-/low- and high-/high-risk HPV genotypes was 63.9 %. The most frequent high-risk genotypes were HPV52 HPV58 and HPV51, whereas the most frequent low-risk genotypes were HPV6, HPV53 and HPV84. Infected women were significantly younger and have less stable partner relationships than uninfected women ($p < 0.05$). Conclusion: A relevant frequency of mixed infections with high- and low-risk HPV genotypes, other than those consi-

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dered most prevalent worldwide, was observed. Most circulating high-risk genotypes among the women of this study are not covered by commercial vaccine formulations.

Keywords

Human Papillomavirus, High-Risk Genotypes, HPV Co-Infections, Linear Array Genotyping

1. Introduction

Strong association between female genital infection with any of the 15 recognized high-risk HPV genotypes and development of cervical cancer has been well established [1]-[3]. Besides high-risk HPV infection, other factors such as promiscuity, weak immune responsiveness, genetic background, long term tobacco smoking and use of oral contraceptives influence malignant transformation [3] [4]. Low-risk HPV genotypes are more commonly associated with warts and low-grade squamous intraepithelial lesions (LSIL) [2] [5].

A latent or persistent infection caused by several HPV genotypes is more likely to occur during pregnancy, even with no evidence of clinical signs. Estimations indicate that less than 10% of HPV-infected women will suffer life-long persistent infection [6].

Since a high proportion of HPV infection is subclinical, cytological and histo-pathological examinations are of restricted clinical value. Use of improved and faster detection/typing methods of HPV in the clinical settings, has allowed identification of women with mild lesions which are prone to evolve into high-grade lesions or carcinomas [6]-[9].

After suspicion of HPV infection, secondary to the main gynecological-obstetric condition of patients, intervention measures at the Mexico's National Institute of Perinatology are restricted to confirmation by colposcopy and/or histo-cytologic examinations. If required, patients are referred to specialized oncologic health services [10].

Considering the above mentioned, the aim of this study was to assess the frequency of HPV genital infection among women with antecedents of HPV infection but without cervical lesions, as well as to identify the circulating HPV genotypes among that group of women at the National Institute of Perinatology in Mexico City.

2. Material and Methods

2.1. Patients

This was a cross-sectional and descriptive study of HPV infection among a group of 145 women without colposcopic evidence of cervical lesions, aged 15 to 68 years. The study was carried out between January and December 2012. Participants were non-randomly selected on convenience basis according to clinical criteria for past or suspected HPV infection, at the National Institute of Perinatology in Mexico City. All women sought primarily gynecologic/obstetric care. The study was approved by the Institutional Research and Ethics Committee (Protocol no. 212250-22761). Informed consent was obtained from all individual participants.

2.2. Samples

Cervical specimens were retrieved by thorough swabbing of the exo- and endo-cervix regions, and collected into PreservCyt™ transport solution-containing vials (Marlborough, Mass., USA). Cervical specimens were kept frozen at −20˚C until processing. Simultaneously, each patient was asked to answer a clinical and socio-demographical questionnaire.

2.3. HPV Genotyping

We used the qualitative "Linear Array HPV Genotyping Test" (Roche Molecular Systems Inc., Branchburg, NJ), according to manufacturer's instructions. This test is designed for specific simultaneous detection and typing of up to 37 HPV genotypes usually found in the human anogenital region, including 13 and 24 of the most frequent

high- and low-risk genotypes, respectively.

Total DNA was extracted from each specimen by treatment with a proteinase K-containing alkaline lysis buffer, and immediately purified by spin sepharose column chromatography. (Qiagen GmbH, Hilden, Germany). Ten-microliter aliquots of the purified DNA samples were subjected to PCR amplification in a 100 uL volume of reaction mix containing 50 μL of 2X master mix, 40 μL of deionized water, and two primers sets, one specific for the HPV L1 gene and the other specific for the human β-globin gene (as quality control of purified DNAs). Amplification was started by incubation at 50˚C/2 min and denaturation at 95˚C/9 min, followed by 40 cycles of denaturation at 95°˚C/30 s-annealing at 55˚C/1 min - extension at 72˚C/1 min, and a final extension at 72˚C/10 min. Positive and negative controls were included in each assay run. After amplification, samples were chemically denatured and poured individually onto cellulose strips with separately blotted recombinant DNA sequences from 37 HPV genotypes. Hybridization was detected by a colorimetric technique directly onto the strips.

2.4. Data Analysis

Socio-demographical and/or frequencies of genotypes data from HPV-infected and uninfected women were statistically compared by using the SPSS v.20 for Windows (IBM, New York, USA). Continuous data were analyzed through Mann-Whitney U or median tests, while analyses of proportions were done by square chi tests.

3. Results

One hundred and forty three cervical specimens gave valid results in the Linear Array HPV Genotyping Test, while two specimens failed to amplify internal control (β-globin gene) and thus were ruled out from further analysis. At least one HPV genotype was detected in 66 specimens (46.2%), while no HPV DNA was detected in 77 (53.8%).

Analyses of the socio-demographic features revealed that HPV-infected women were significantly younger ($p = 0.012$) and apparently spent fewer years for education than women without HPV infection ($p = 0.058$, **Table 1**). In addition, a higher proportion of women without HPV infection seem to have long term partner relationships than infected women ($p = 0.015$). The number of sex partners and the age at which women became sexually active did not differ considerably between HPV-infected and uninfected subgroups.

The comparison of socio-demographic features between single- and multiple-genotype HPV-infected women revealed no significant differences (**Table 2**), even though women in the latter group had slightly more sex partners.

Regarding detection/genotyping, a total of 112 positive HPV signals were obtained among the study group, corresponding to 28 out of the 37 detectable genotypes by the test used here. Approximately one third of all viruses detected were identified as high-risk HPV genotypes, whereas the remaining viruses were identified as low-risk genotypes, whose individual frequencies are shown in **Figure 1**. Overall, the most frequent genotypes among study group were HPV6, 52, 53 and 58.

Figure 2 shows multiple-genotype infections (with up to four genotypes) in single samples. Noteworthy these multiple-genotype infections were slightly more frequent than single-genotype infections (54.5% vs. 45.5%, respectively). Interestingly the two-genotype HPV infections were the more frequent form of multiple-genotype infection, either of the same oncogenic risk category or of combined risk categories (**Table 3**).

The low-risk HPV6 was the predominant genotype among patients with single-genotype infection, followed by low-risk HPV53 and high-risk HPV16. The approximate ratio of low-risk to high-risk genotypes among the single-genotype infected women was 2:1 (**Table 3**). The predominant combination of multiple HPV genotype infection was that of HPV52 plus HPV58 genotypes, reaching 13.9% (**Table 3**).

4. Discussion

Rise in worldwide HPV prevalence among women, and consequently a rise in cases of cervical cancer (from 378,000 cases per year in 1980 to 454,000 cases per year in 2010), has been observed in the past 2 decades, although it varies geographically, and according to age and other factors [11]-[15].

On one hand, it is undeniable that ultimate generation DNA extraction/purification and amplification methods have had an enormous impact on improving detection of infectious agents in the clinical setting. On the other

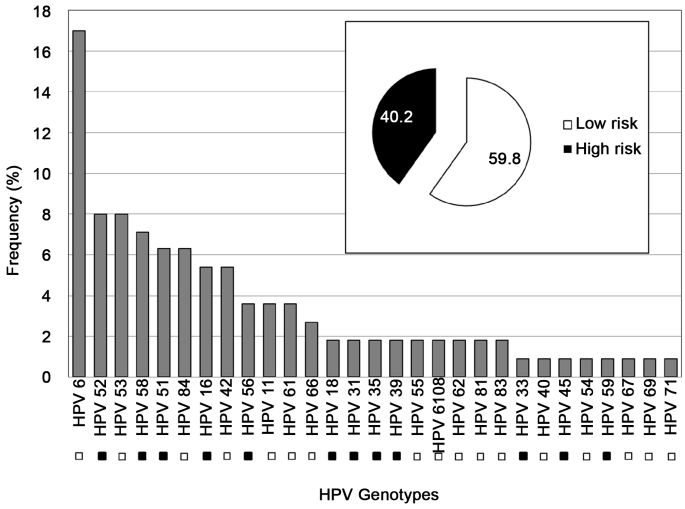


Figure 1. Oncogenic risk and frequencies of HPV genotypes.

Table 1. Socio-demographic features of women in this study.

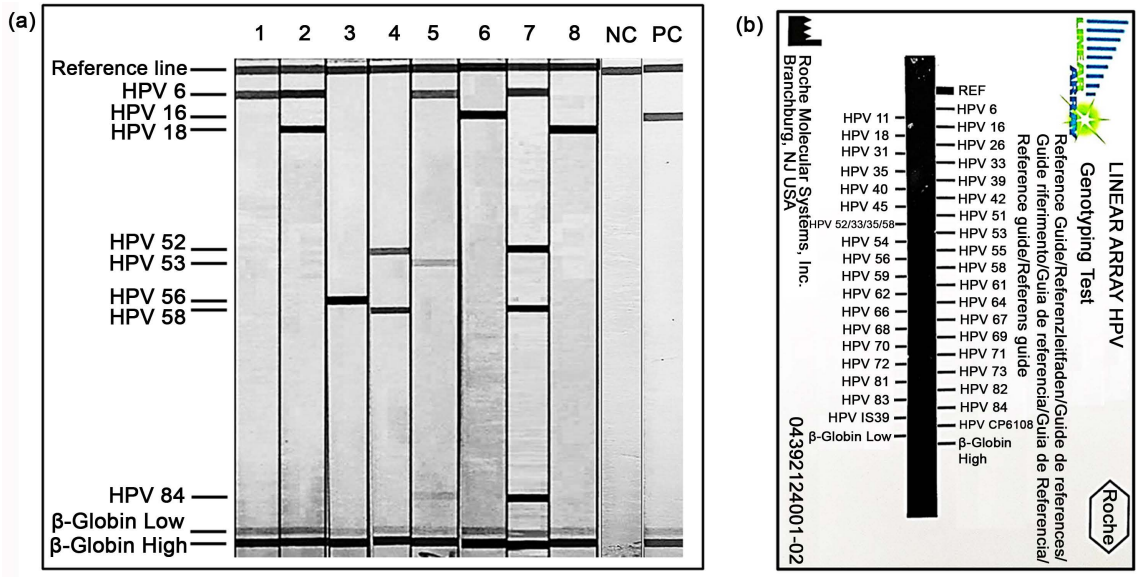
Variable	HPV status		
	Infected n = 66	Non Infected n = 77	p value[†]
***Age* (*years*)**			
Mean ± SD (Standard Deviation)	28.6 ± 9.0	33.7 ± 10.1	0.012[‡]
Median	26.5	31.0	NS[§]
≤21, No. (%)	16 (24.2)	8 (10.4)	0.027[¶]
>21, No. (%)	50 (75.8)	69 (89.6)	
***Age at first sexual Intercourse* (*years*)**			
Mean ± SD	19.2 ± 4.6	19.4 ± 4.2	NS[‡]
Median	18.0	19.0	NS[§]
≤16, No. (%)	23 (34.8)	17 (22.1)	NS[¶]
>16, No. (%)	43 (65.2)	60 (77.9)	
Number of sex Partners			
Mean ± SD	3.5± 4.1	2.7± 2.2	NS[‡]
Median	2.0	2.0	NS[§]
Range	1 - 20	1 - 15	
Single, No. (%)	20 (30.3)	21 (27.3)	NS[§]
Multiple, No. (%)	46 (69.7)	56 (72.7)	
***Education* (*years*)**			
Mean ± SD	10.6 ± 2.8	11.9 ± 3.8	NS[‡]
Median	10.5	12.0	0.011[§]
≤9, No. (%)	33 (50.0)	30 (39.0)	NS[‡]
>9, No. (%)	33 (50.0)	47 (61.0)	
Stable partner relationship			
Yes, No. (%)	36 (54.5)	57 (74.0)	0.015[¶]
No, No. (%)	30 (45.5)	20 (26.0)	
Occupation			
Unpaidworker, No. (%)	56 (84.8)	59 (76..6)	NS[¶]
Wageworker, No. (%)	10 (15.2)	18 (23.4)	

[†]Significance value set al $p < 0.05$; NS, Not significant. [‡]Mann-Whitney U test for independent samples comparing the mean values of continuous variables. [§]Median Test for Independent Samples. [¶]Square Chi Test and/or Fisher's Exact Test comparing proportions of patients with a given variable.

Table 2. Comparison of socio-demographic features among HPV-infected women.

Variable	Type of HPV infection		
	Single genotype (n = 30)	Multiple genotype (n = 36)	p value[†]
***Age* (*years*)**			
Mean ± SD (Standard Deviation)	28.6 ± 9.0	33.7 ± 10.1	NS[‡]
Median	26.5	26.5	NS[§]
≤21, No. (%)	6 (20.0)	10 (27.8)	NS[¶]
>21, No. (%)	24 (80.0)	26 (72.2)	
***Age at first sexual Intercourse* (*years*)**			
Mean ± SD	19.1 ± 3.8	19.3 ± 5.3	NS[‡]
Median	18.0	17.0	NS[§]
≤16, No. (%)	8 (26.7)	15 (41.7)	NS[¶]
>16, No. (%)	43 (65.2)	60 (77.9)	
Number of sex Partners			
Mean ± SD	2.9 ± 2.7	4.0 ± 5.0	NS[‡]
Median	2.0	2.0	NS[§]
Range	1 - 6	1 - 20	
Single, No. (%)	9 (30.0)	11 (30.6)	NS[§]
Multiple, No. (%)	21 (70.0)	25 (69.4)	
***Education* (*years*)**			
Mean ± SD	10.5 ± 2.9	10.8 ± 2.8	NS[‡]
Median	9.0	12	NS[‡]
≤9, No. (%)	17 (56.7)	16 (44.4)	NS[‡]
>9, No. (%)	13 (43.3)	20 (55.6)	
Stable partner relationship			
Yes, No. (%)	16 (53.3)	20 (55.6)	0.015[¶]
No, No. (%)	14 (46.7)	16 (44.4)	
Occupation			
Unpaidworker, No. (%)	25 (83.3)	31 (86.1)	NS[¶]
Wageworker, No. (%)	5 (16.7)	5 (13.9)	

[†]Significance value set al $p < 0.05$; NS, Not significant. [‡]Mann-Whitney U test for independent samples comparing the mean values of continuous variables. [§]Median Test for Independent Samples. [¶]Square Chi Test and/or Fisher's Exact Test comparing proportions of patients with a given variable.



Line 1: HPV6; **Line 2:** HPV6, 18; **Line 3:** HPV56; **Line 4:** HPV52, 58; **Line 5:** HPV6, 53, 84; **Line 6**: HPV16; **Line 7:** HPV6, 52, 58, 84; **Line 8:** HPV18; **Line 9:** C(-), **Line: 10:** C(+/HPV16).

Figure 2. Selected profile of genotyping results.

Table 3. Single- and multiple-genotype HPV infections among the study group.

Number of genotypes	No. (%)	Oncogenic Risk			% of study group (n = 143)
		High	Low	Mixed	
		GTs[‡] (No.)[§]	GTs[‡] (No.)[§]	GTs[‡] (No.)[§]	
1	30 (45.5)	16 (2) 18 (1) 39 (1) 45 (1) 51 (2) 56 (1) 59 (1)	6 (9) 42 (1) 53 (5) 55 (1) 61 (2) 62 (1) 66 (1) 84 (1)		21
2	28 (42.4)	16 + 35 (1) 35 + 52 (1) 52 + 58 (5)	6 + 53 (1) 6 + 55 (1) 6 + 6108 (2) 11 + 66 (1) 42 + 54 (1) 42 + 84 (1) 53 + 83 (1) 61 + 71 (1) 61 + 84 (1) 83 + 84 (1)	16 + 81 (1) 18 + 6 (1) 33 + 11 (1) 39 + 6 (1) 51 + 11 (1) 51 + 69 (1) 52 + 42 (1) 56 + 40 (1) 56 + 67 (1) 56 + 84 (1)	19.6
3	6 (9.1)	16 + 51 + 58 (1)	6 + 53 + 84 (1) 6 + 62 + 81 (1)	16 + 51,+ 53 (1) 31 + 42 + 84 (1) 52 + 58 + 6 (1)	4.2
4	2 (3.0)			31 + 51 + 11 + 42 (1) 52 + 58 + 6 + 66 (1)	1.4
Totals	**66 (100)**				**41.2**

[†]Per single patient. [‡]HPV genotypes. [§]Numbers within parenthesis indicate frequency of cases.

hand, there is an ongoing openness in sexual practices among teenagers and young adults, who by the way are not afraid anymore of HIV/AIDS or other sexually transmitted infections, which in turn increases the likelihood for acquisition of HPV [16] [17]. Therefore it appears that such increase in cases of HPV-associated cervical cancer is the result of the concerted occurrence of the above mentioned issues.

In this study less than 50% of the women were infected with one or more HPV genotypes, which were heterogeneously distributed irrespective of their oncogenic risk. Our results are comparable with those from other studies with Mexican women [11] [18] [19]. In contrast, a meta-analysis about worldwide HPV detection among women with normal cytological findings, revealed an estimated frequency for Central America (including Mexico) of around 20%, while estimated frequencies for worldwide and developing region were 7.2% and 14.3%, respectively [20].

Data presented here revealed that HPV-infected women were significantly younger than those of the uninfected group, in agreement with previous reports [2] [18] [20] [21]. In contrast with results from other study with Mexican women [12], we do not found significant association between HPV infection and the number of sex partners, even though women infected with multiple genotypes had more sex partners than those infected by a single genotype, though this was not statistically significant. Lower level of education, short-term or null partner relationships and unpaid work were the predominant socio-demographic issues among HPV-infected women. Along with low income, those variables are highly associated with acquisition of HPV infection and/or cancer development [14] [18] [22] [23].

In this work, the relatively uncommon HPV genotypes in the cervix, HPV6, 52, 53, 58, 51 and 84, were detected at the highest frequencies (ranging from 17% to 6.3%). The main oncogenic genotypes, HPV16 and HPV18, were detected in frequencies lower than expected (5.4% and 1.8%, respectively). In contrast, higher frequencies of genotypes HPV16, 18 and 58 were reported among women from the southern coastal regions of Mexico, (14.6 %, 8.2% and 7.9%, respectively), whereas low-risk genotypes HPV 11 and 53 were found in frequencies below 6.8% [11]. In a survey carried out among 1340 women of the South Central region of Mexico, whose cytological findings were normal, the high-risk genotypes HPV 16, 53, 31 and 18 were the most common, but their individual frequencies were lesser or equal to 1.7% [12].

The above mentioned studies used two different experimental approaches, one used universal PCR coupled to direct sequencing and the other used a reverse-line blot assay for up to 27 genotypes, which partially explain the differences between these and our study. A meta-analysis study showed that genotypes HPV16, 18, 31, 52, and 58 are consistently found among the 10 most common types across world regions, among women with normal cytological findings [18].

On the contrary, when groups of Mexican women presenting LSIL, HSIL or cervical cancer were analyzed, frequencies of HPV infection increased from 42% to 95%, irrespective of the lesion type [19], although individual high-risk genotype frequencies were variable between studies. This confirms that there is a high correlation between presence of oncogenic HPV genotypes and different cervical lesions [2] [4]. In our study women had no evidence of cervical lesions and this partially explains the differing frequencies of high-risk genotypes.

Despite there are evident coincidences in the HPV genotypes identified in several studies, the use of different experimental approaches hampers appropriate comparisons. This confirms the need to uniform diagnostic approaches with the best molecular assay available, this should include identification of the maximum number possible of circulating HPV genotypes, without restricting detection to high-risk ones [6]-[9].

Nearly 60% of all HPV-infected patients in our study had multiple infections, with up to 4 genotypes, generally high- and low-risk types; this finding is in agreement with other studies employing the same molecular assay [6] [8] [24] [25]. Even though the presence of low-risk HPV genotypes within genital warts and tissue samples from cervical cancer has been demonstrated [2] [4] [12], there is a pervasive notion among clinicians that infection with low-risk HPV genotypes will not trigger malignant transformation, thus they neglect this finding as a threat for carcinogenesis [26]. The likelihood that those viruses with varying oncogenic potential can compete or act synergistically is rather high [4] [26] [27]. Moreover it is possible that simultaneous presence of 2 or more HPV genotypes can render a given patient more susceptible to develop cervical lesions than those who have a single-genotype infection.

Therefore, we strongly suggest mid- to long-term follow up for women presenting high- and low-risk genotypes, in order to detect and prevent further complications. Additionally, the varying geographical distribution of HPV types, also with diverse oncogenic potential [2] [28]-[30], suggest the importance of population-based studies to assess the influence of local genotypes prevalence on vaccine efficacy for the prevention of cervical cancer.

The relative high frequency of multiple-genotype infection among the women in our study could be sugges-

tive of either persistence of some genotypes and subsequent acquisition of other genotypes (through risky sexual practices), or increased age-related host susceptibility [26].

In the light of the frequent multiple HPV infections detected in this study, some reports have suggested an association between multiple-genotype HPV infection and presence of cervical intraepithelial neoplasia and/or cervical cancer [27]. Accordingly, identification of both high- and low-risk HPV genotypes should be performed routinely, since an important proportion of cytology examinations will not show abnormal findings [26].

5. Conclusion

The use of a test able to detect/type up to 37 HPV genotypes, unrestricted to high-risk genotypes, allowed the identification of a relevant frequency of multiple HPV infections among a group of Mexican women without cervical abnormalities. Whether co-infections with low-risk HPV genotypes, or with high- and low-risk genotypes, may progress to pre-malignant or malignant transformation remains to be determined.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Ethical Approval

Research was conducted according to ethical principles, and was approved by the Institutional Research and Ethics Committee (Protocol no. 212250-22761) of National Institute of Perinatology, México City, México.

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