MAJOR ARTICLE

Cervical Coinfection with Human Papillomavirus (HPV) Types and Possible Implications for the Prevention of Cervical Cancer by HPV Vaccines

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Coinfection with multiple types of human papillomavirus (HPV) and its implications for the development of efficacious HPV vaccines is a subject of great interest. To describe the occurrence of concurrent infection with multiple HPV types and to determine whether genital HPV infection modifies the risk of acquiring a new HPV infection with another HPV type, 1610 subjects were monitored for an average of 4.1 years in Bogotá, Colombia. Information on risk factors for HPV infection and cervical cells was collected for detection of HPV DNA of 36 types at study entry and at 6 consecutive 6-month follow-up visits. Clustering or the concurrent acquisition of multiple types occurred more often than would be expected by chance. Subjects with incident HPV-16 or -18 infection had 5–7 times higher odds of acquiring a subsequent HPV-58 infection than subjects not infected with HPV-16 or -18. This might affect the protection conferred by effective HPV vaccines.

Accumulated evidence has shown that genital infection with ⩾1 of ~15 human papillomavirus (HPV) types is a necessary cause of cervical cancer [1, 2], which has led to the design of prophylactic vaccines. Although there is some evidence of cross-reactivity among certain HPV types [3], it is accepted that effective HPV vaccines should contain the types responsible for most cervical cancers [4]. It has been speculated, however, that the elimination of certain HPV types by vaccination might lead to changes in the distribution of other types by enhancing or decreasing the risk of infection. Studies of the natural history of cervical HPV infections could provide clues to explore the impact of vaccination on other types, because 20%–30% of HPV-infected women harbor multiple types that were acquired concurrently or sequentially.

In a study of young women from the United States, preexisting HPV-16 infection was associated with an increased risk of the subsequent acquisition of other HPV types [5]. In a second study of college students in the United States, the concurrent acquisition of multiple HPV types occurred more often than would be expected by chance, but those authors failed to identify types that were more likely to be detected together [6]. In another study in Brazil, the acquisition of a new infection was more likely among women with any HPV type detected at study entry, and persistence was independent of coinfection with other types [7].

We report here on coinfection with different HPV types in an ongoing cohort of Colombian subjects. The main objective was to determine whether subjects infected with a specific HPV type were at a different risk of acquiring a new infection with a phylogenetically related or unrelated HPV type than were those not in-

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fected with the index type and to make predictions regarding the impact of vaccination on HPV types not contained in the vaccine.

SUBJECTS, MATERIALS, AND METHODS

Study population and follow-up. Between November 1993 and November 1995, a total of 2200 sexually active subjects who presented to family-planning clinics and cervical cancer screening centers were invited to participate in a prospective study of cervical HPV infection. Eligible subjects were females ⩾13 years old who resided in Bogotá, Colombia; had no history of preneoplastic or neoplastic lesions of the cervix or of conization or hysterectomy; and were willing to provide informed consent. At entry, participants completed a questionnaire and underwent a pelvic examination for the collection of cervical cells for cytological testing and the detection of HPV DNA. Follow-up visits were scheduled every 6 months thereafter; we present data for the first 6 visits, up to December 1999, for almost 80% ($n = 1720$) of the subjects and up to 2001 for the remaining 20%. At each visit, a questionnaire on lifestyle and sexual behavior was administered, a pelvic examination was performed, and cervical specimens were collected for cytological assessment and HPV detection.

Of the 2200 subjects who were invited to participate in the study, 53 (2.4%) refused participation, 8 (0.4%) were considered to be ineligible (because of a history of cervical cancer, hysterectomy, or mental illness), 29 (1.3%) did not provide adequate specimens for HPV detection, 94 (4.3%) had inadequate HPV test results because of poor DNA quality (i.e., failure to amplify the β-globin gene or a negative HPV test), and 147 (6.7%) had abnormal cytological results at baseline. In addition, 12 (0.5%) subjects <15 years old contributed data only from a single visit and were excluded, leaving 1857 subjects. When specimens collected at baseline were used, 247 subjects tested positive for HPV, leaving 1610 as the study population.

HPV detection. Testing for HPV was conducted by use of a standard $GP5^+/GP6^+$ polymerase chain reaction (PCR)–EIA [8]. Briefly, HPV-positive samples were subjected to EIA-HPV group-specific analysis by use of probe mixtures for high-risk and low-risk HPVs [9]. The high-risk HPV probe mixture consisted of oligoprobes for HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68; the low-risk HPV probe consisted of oligoprobes for HPV-6, -11, -26, -34, -40, -42–44, -53–55, -57, -61, -70–73, -81–84, and CP6108. The low-risk probe mixture contained HPV-73 and -82, which have been classified as high-risk types and some HPV types (26, 34, and 53), that have been classified as probably high-risk types [3]. These types were classified as high-risk.

Additionally, HPV positivity was assessed by Southern-blot hybridization of $GP5^+/GP6^+$ PCR products with the general probe of specific DNA fragments from cloned DNA of HPV-6, -11, -16, -18, -31, and -33 under low stringent conditions [10]. Samples that were positive by Southern-blot analysis and negative by high-risk/low-risk EIA were considered to be HPV-X, or of undetermined type, and were classified as low-risk.

During follow-up, a new $GP5^+/GP6^+$ PCR reverse-line blot analysis (PCR-RLB) was developed; this was used to type the same 36 different HPV types as those detected by PCR-EIA. Specimens collected during the first 4 visits were typed by PCR-EIA, and those collected during visits 5 and 6 were typed by PCR-RLB. Agreement between PCR-RLB and PCR-EIA was found in 96% of cases [11].

Statistical methods. The longitudinal data for each participant were assembled in as many pairs of consecutive visits (i.e., index and follow-up visits) as the subject provided. The time lag between visits of each pair was restricted to 3–18 months.

For the event of interest being incidence of infection with any HPV type, we used the pairs for which the index visit resulted in negative tests for all types. The outcome of interest was the number of the individuals (y_x) at the follow-up visit who tested positive for 0, 1, 2, … , HPV types among the 36 different types. Under the null hypothesis of no clustering among HPV types, y_x follows a Poisson distribution with mean and variance equal to m. Under the alternative hypothesis of clustering, y_x will have a variance greater than the mean and will be more appropriately described by a negative binomial distribution whose variance is $m(1 + ms^2)$, where s is the parameter describing the magnitude of the clustering of HPV types. An estimate of s can be obtained by the square root of {[var (y)/mean (y)] − 1}/mean (y), where mean (y) and var (y) are the mean and variance, respectively, of y_x. To test the null hypothesis of no clustering (H_0: $s = 0$), we used maximum-likelihood methods, in particular the likelihood ratio test. The type-specific incidence of high- and low-risk types was determined by use of similar methods, but subjects could test positive at the index visit for other types (e.g., for low-risk types at the index visit when we analyzed the clustering of high-risk types).

Another measure of clustering was provided by the odds ratio (OR) of incident concurrent infections with 2 HPV types. We focused attention on types belonging to the phylogenic group A7, which includes HPV-16, -31, -33, -52, and -58, and to the group A9, which includes HPV-18, -39, and -45. The pairs relevant to coinfection with 2 types were from those who tested negative at the index visit for the 2 types of interest. The outcome of interest was the infection status at the follow-up visit and was given as −/−, +/−, −/+, and +/+ for the 2 types (in which "−" indicates a negative result and "+" indicates a positive result); ORs were calculated as the cross-product of the number of subjects for each infection status. We determined the role played by HPV infection as a risk factor for infection with another HPV type by use of paired visits as the units of analysis, with the only restriction that the index visit be negative

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Table 1. Observed and expected counts of new infections with different human papillomavirus (HPV) types.

No. of HPV types per visit	Any HPV		High-risk HPV		Low-risk HPV	
	Observed counts (%) (n = 4193 visits)	Expected counts (no clustering)	Observed counts (%) (n = 4290 visits)	Expected counts (no clustering)	Observed counts (%) (n = 4529 visits)	Expected counts (no clustering)
0	3935 (93.9)	3875.66	4076 (95.0)	4045.26	4434 (97.9)	4417.40
1	203 (4.8)	305.01	181 (4.2)	237.62	82 (1.8)	110.22
2	43 (1.0)	12.00	29 (0.7)	6.98	8 (0.2)	1.37
3	9 (0.2)	0.31	3 (0.1)	0.14	5 (0.1)	0.01
4	2 (0.1)	0.01	1 (0)	<0.01	0 (0)	<0.01
5	0 (0)	<0.01	0 (0)	<0.01	0 (0)	<0.01
6	1 (<0.1)	<0.01	0 (0)	<0.01	0 (0)	<0.01
2–6	55 (1.3)	12.32	33 (0.8)	7.12	13 (0.3)	1.39
Distribution						
Mean	0.0787	0.0787	0.0587	0.0587	0.0249	0.0249
Variance	0.1188	0.0787	0.0758	0.0587	0.0345	0.0249
Clustering[a]	2.54[b]	0[b]	2.22	0	3.91[b]	0

[a] Clustering was determined by the formula $\{[(\text{variance/mean}) - 1]/\text{mean}\}^{1/2}$.
[b] $P < .001$.

for the specific type of interest whose positivity at the follow-up visit was the outcome.

The primary exposure was the presence of another type at the index visit, and the outcome was whether the subject tested positive for the virus of interest at the follow-up visit. For example, among the pairs of visits that yield negative test results for HPV-18 at the index visit, we investigated whether those who at the index visit were positive for HPV-16 were more likely to test positive for HPV-18 at the follow-up visit. To measure the association, we used standard methods for ORs and logistic regression to adjust for multiple factors. Of particular interest in this analysis was the determination of whether positivity at the index visit for any of the 4 types included in a proposed vaccine (i.e., HPV-16, -18, -6, and -11) indicated a predisposition for testing positive at the follow-up visit for each 1 of the 8 most common types mentioned above. Estimates were adjusted for age, number of lifetime sex partners at baseline, and new sex partners during follow-up, to attempt to equalize for risk profile. Because each woman could contribute repeated measurements over time, inferences were based on robust statistical methods [12] that adjusted for the correlation inherent in such repeated measurements.

RESULTS

A total of 1610 female subjects, 15–85 years old, with normal cytological results and a negative HPV test at study entry were included in the analysis. The median duration of follow-up was 4.1 years (interquartile range [IQR], 3.2–5.0 years), and the median interval between visits was 7 months (IQR, 6.0–12.0 months). More than 65% of subjects made at least 4 visits (IQR, 2.0–5.0 visits).

The baseline characteristics of the subcohort were very similar to those of the entire cohort, which has been described elsewhere [8]. The median age was 32.3 years (IQR, 26.5–39.2 years); all subjects were sexually active, and 20% reported having had >1 regular sex partner; the median number of lifetime full-term pregnancies was 2 (IQR, 1–3 pregnancies); 47.5% had ever used oral contraceptives before the baseline visit; 18.6% were current smokers, and 11.2% were former smokers.

The incidence rate of infection with any HPV type in the total cohort was 6.2 cases/100 woman-years. Specific incidence rates are described in detailed elsewhere [13].

The 1610 subjects contributed 4912 pairs of visits during the follow-up. In 4193 (85.4%), 4290 (87.3%), and 4529 (92.2%) pairs, the index visits showed negative results for any type, high-risk types, and low-risk types, respectively (table 1). Of the 316 subjects with incident infections during follow-up, 258 (81.6%) had an infection within the time span of our paired analysis. Among these, 21.3% (55/258) showed concurrent infections by >1 HPV type. The maximum number of high-risk or low-risk types observed in a single incident infection was 6. Observed counts of concurrent infections by any HPV type were found in all cases to be significantly different, compared with the expected distribution under the assumption that there was no clustering ($P < .001$). For example, 43 visits with double incident coinfections were observed, whereas only 12 were expected under the assumption of no clustering; also, 9 visits with infection with 3 new HPV types were observed, whereas <1 was expected under the assumption of no clustering. Similar results were observed for high-risk and low-risk types, as shown in table 1.

Table 1 documents the presence of a significant clustering of infection with multiple types. For any HPV, high-risk, and low-risk incident infections, the observed number of subjects infected with >1 HPV type were 4.5 (55/12.32), 4.6 (33/7.12),

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and 9.4 (13/1.39) times the corresponding expected count under the assumption of no clustering. Indeed, the magnitude of clustering of HPV types was 2.54, 2.22, and 3.91 for any HPV, high-risk types, and low-risk types, respectively, with each being strongly significant ($P < .001$).

The adjusted ORs of concurrent infection with pairs of different HPV types ranged from 3 to 25 (table 2). Significant positive associations were observed both within and between HPV types of phylogenetic groups A7 and A9. We were not able to estimate some ORs, because of the small number of visits with coinfection with specific combinations of HPV type. However, when we adjusted for age and lifetime number of sex partners, results suggested that the risk of concurrent infection was significantly increased for most pairs of HPV types evaluated. For example, subjects with a new infection with HPV-18 had 12.1 times the odds of having a concurrent infection with HPV-39, 17.7 times the odds of having a concurrent infection with HPV-45, and 11.4 times the odds of having a concurrent infection with HPV-31 than subjects not infected with HPV-18. In contrast, we found lower and nonsignificant associations for specific paired combinations between HPV-18 and HPV-16, -52, and -58.

Estimates of changes in the probability of infection at a subsequent (i.e., follow-up) visit according to HPV type–specific

Table 2. No. of pairs and odds ratios (ORs) of incident concurrent infection according to pairwise combinations of specific human papillomavirus (HPV) types.

	HPV-18		HPV-39		HPV-45		HPV-16		HPV-31		HPV-33		HPV-52	
HPV type, ORs	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
Group A9														
HPV-39														
Yes	1	36												
No	20	5498												
Crude OR	7.6													
Adjusted OR[a]	**12.1**													
HPV-45														
Yes	3	25	0	28										
No	35	5479	23	5522										
Crude OR	**18.8**		NE											
Adjusted OR	**17.7**		NE											
Group A7														
HPV-16														
Yes	2	54	5	52	1	56								
No	34	5353	17	5406	27	5373								
Crude OR	**5.8**		**30.6**		3.6									
Adjusted OR	3.5		**12.8**		NE									
HPV-31														
Yes	3	32	0	36	2	34	0	35						
No	34	5458	23	5502	25	5482	57	5356						
Crude OR	**15.0**		NE		**12.9**		NE							
Adjusted OR	**11.4**		NE		**12.1**		NE							
HPV-33														
Yes	0	16	0	17	0	17	1	15	2	15				
No	38	5438	21	5537	28	5509	55	5394	31	5503				
Crude OR	NE		NE		NE		6.5		**23.7**					
Adjusted OR	NE		NE		NE		5.8		6.5					
HPV-52														
Yes	1	25	1	26	1	26	2	26	1	26	0	28		
No	37	5473	21	5526	27	5501	54	5379	35	5483	17	5511		
Crude OR	5.9		10.1		7.8		**7.7**		6.0		NE			
Adjusted OR	8.8		13.1		NE		**9.0**		**6.2**		NE			
HPV-58														
Yes	1	38	1	40	0	41	1	37	3	38	4	34	0	41
No	35	5425	22	5475	29	5447	53	5337	33	5433	12	5483	27	5451
Crude OR	4.1		6.2		NE		2.7		**13.0**		**53.8**		NE	
Adjusted OR	5.8		**10.6**		NE		3.3		NE		**25.0**		NE	

NOTE. Bold type, $P < .05$; NE, not estimable because there were no cases with specific combinations; regular type $P \geq .05$.

[a] OR adjusted for age, no. of lifetime sex partners, and new sex partners during follow-up.

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Table. 3. Percentage of pairs who tested positive at index visits for human papillomavirus (HPV) type and odds ratios (ORs) for incident infections with types.

	OR of incident HPV type at follow-up visit															
	HPV-18		HPV-39		HPV-45		HPV-16		HPV-31		HPV-33		HPV-52		HPV-58	
HPV type at index visit	Yes (n = 38)	No (n = 5539)	Yes (n = 23)	No (n = 5592)	Yes (n = 29)	No (n = 5566)	Yes (n = 57)	No (n = 5444)	Yes (n = 36)	No (n = 5549)	Yes (n = 17)	No (n = 5581)	Yes (n = 28)	No (n = 5569)	Yes (n = 41)	No (n = 5519)
Group A9																
HPV-18			8.70	1.04	3.45	0.93	1.75	1.07	2.78	1.03	5.88	1.08	7.14	1.06	4.88	1.07
Crude OR	NA	NA	**9.1**		**3.8**		1.7		2.8		5.8		**7.2**		**4.7**	
Adjusted OR[a]			NE		4.7		NE		3.5		6.7		**12.5**		**6.8**	
HPV-39	2.63	0.38			3.45	0.38	0.00	0.39	0.00	0.43	0.00	0.41	3.57	0.40	0.00	0.40
Crude OR	7.1		NA	NA	**9.4**		NE		NE		NE		**9.3**		NE	
Adjusted OR	NE				NE		NE		NE		NE		NE		NE	
HPV-45	0.00	0.63	0.00	0.75			0.00	0.81	0.00	0.76	0.00	0.79	3.57	0.74	0.00	0.78
Crude OR	NE		NE		NA	NA	NE		NE		NE		5.0		NE	
Adjusted OR	NE		NE				NE		NE		NE		5.3		NE	
Group A7																
HPV-16	5.26	2.38	4.35	2.40	3.45	2.16			2.78	2.45	5.88	2.37	0.00	2.44	7.32	2.34
Crude OR	2.3		1.9		1.4		NA	NA	1.1		2.6		NE		**3.3**	
Adjusted OR	3.3		NE		NE				NE		2.6		NE		**4.5**	
HPV-31	2.63	0.88	0.00	0.97	6.90	0.90	0.00	0.97			0.00	0.84	3.57	0.92	0.00	0.96
Crude OR	3.0		NE		**8.2**		NE		NA	NA	NE		4.0		NE	
Adjusted OR	NE		NE		NE		NE				NE		5.7		NE	
HPV-33	0.00	0.72	8.70	0.68	3.45	0.72	1.75	0.64	8.33	0.56			0.00	0.74	7.32	0.43
Crude OR	NE		**13.9**		4.9		2.8		**16.2**		NA	NA	NE		**18.1**	
Adjusted OR	NE		NE		NE		NE		NE				NE		NE	
HPV-52	0.00	0.74	4.35	0.72	3.45	0.70	1.75	0.72	0.00	0.72	0.00	0.75			0.00	0.74
Crude OR	NE		6.3		5.1		2.5		NE		NE		NA	NA	NE	
Adjusted OR	NE		**10.2**		**9.2**		3.5		NE		NE				NE	
HPV-58	5.26	1.37	0.00	1.32	0.00	1.40	5.26	1.29	0.00	1.41	5.88	1.15	3.57	1.38		
Crude OR	4.0		NE		NE		**4.3**		NE		5.4		2.6		NA	NA
Adjusted OR	**6.1**		NE		NE		**5.9**		NE		5.7		NE			

NOTE. Bold type, $P<.05$; NA, not applicable, same HPV type; NE, OR not estimable because there were no cases with the specific combinations; regular type, $P\geq.05$.

[a] OR adjusted for age, no. of lifetime sex partners, and new sex partners during follow-up.

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infection at a previous (i.e., index) visit are shown in table 3. Adjusted ORs ranged from 2 to 12, and many of them were not significant. Our results support an increased risk of subsequent infection with specific HPV types for previous infection with HPV-18, -16, -52, and -58. For example, subjects who tested positive for HPV-18 at the index visit had 12.5 times the odds of an incident infection with HPV-52, compared with subjects who were HPV-18–negative at the index visit (table 3). Infections with HPV-18 or -16 made a subsequent incident infection with HPV-58 more likely (OR, 6.8 and 4.5, respectively). Also, infection with HPV-52 made a subsequent infection with HPV-39 and -45 more likely, and infection with HPV-58 was associated with subsequent new infection with HPV-18 and -16. Except for the associations observed between HPV-16 and -58 within the A7 phylogenetic group, 5 of 7 significant associations were found between HPV types from a different phylogenetic group (A7 or A9).

We further evaluated the risk of subsequent infections according to the simultaneous presence at the index visits of $\geqslant 1$ of HPV-16, -18, -6, and -11, which have been included in a designed HPV vaccine (table 4). We found that, independent of age and new sex partners, the observed risk of infection with HPV-58 was increased >4 times by a previous infection with $\geqslant 1$ of the types included in the potential vaccine. Also, our data support an increased risk of new HPV-18 infection in the case of previous infection with HPV-6 or -11, the low-risk types included in the vaccine candidate. Using the statistically significant ORs shown in table 4 and the prevalence of infection with any of the types in the designed vaccine, we calculated the percentages of the incident infections with HPV-18 and -58 that might be prevented if the vaccine is to be fully efficacious against infections with $\geqslant 1$ of the other types included. Specifically, 9.3% (95% confidence interval [CI], 1.0%–28.9%) and 13.4% (95% CI, 3.5%–31.8%) of the incident infections with HPV-18 and -58, respectively, might be prevented.

DISCUSSION

We extended previous observations on coinfection with multiple HPV types reported by other researchers [5–7] by including only incident infections occurring in an ongoing cohort study of Colombian subjects and by increasing the number of visits and follow-up periods. We found that 21.3% of 258 incident HPV infections occurred with multiple types and that the acquisition of multiple types occurred more frequently than would be expected by chance, even after adjustment for age and lifetime number of sex partners, which are the main determinants of the acquisition of HPV infection. This observation is in agreement with previous reports [5–7], but here we precisely quantify the level of clustering in all HPV types and in the high-risk and low-risk types.

We detected significant statistical associations in the concurrent and sequential acquisition of several pairs of HPV types. For example, subjects infected with HPV-18 had 11–18 times higher odds of acquiring concurrent infections with HPV-31, -39, and -45 than subjects without HPV-18 infection. Subjects with incident infections with HPV-16 or -18 had 5–7 times higher odds of acquiring a subsequent HPV-58 infection than subjects without those types.

To extend these observations, we assessed the risk of concurrent and sequential coinfection in subjects with incident infections with the HPV types contained in one of the vaccines presently under evaluation (HPV-6, -11, -16, and -18). Our findings may be interpreted in several ways. First, the increased risk of coinfection or subsequent infection could be the result of common exposure or common routes of transmission shared by the 2 HPV types under analysis; we tried to control for this possibility by adjusting for age, number of lifetime sex partners, and new sex partners during follow-up, which are the main determinants of HPV acquisition as previously identified in this population [13]. However, this control might have been insufficient if other unmeasured factors that may also influence the risk of HPV acquisition—such as the sexual behavior of the partners—were not controlled for. Second, subjects who received a vaccine against HPV-16 and -18, in addition to being protected against these 2 types, may also be at a significantly lower risk of being infected with HPV-58. This could be true if there is a biological interaction between these 2 types, such that HPV-16 facilitates infection with HPV-58. This may be the case; it has been reported that these 2 types use the same endocytosis pathway to enter cells [14]. Concerning cross-protection, some cross-neutralization has been observed for HPV-16, -31, and -33 but not for HPV-16 and -58 [3].

Table 4. Odds ratio (OR) of subsequent new infection according to previous infection with $\geqslant 1$ of human papillomavirus (HPV) types contained in an HPV vaccine currently under evaluation.

HPV types at index visit	HPV type at subsequent follow-up visit							
	HPV-18	HPV-39	HPV-45	HPV-16	HPV-31	HPV-33	HPV-52	HPV-58
HPV-16, -18	3.3 (0.7–14.7)	NE	1.2 (0.1–10.2)	NE	0.8 (0.1–6.4)	4.1 (0.8–21.0)	3.3 (0.7–16.3)	**5.7 (2.2–15.1)**
HPV-6, -11	**14.1 (2.1–95.4)**	NE	7.7 (0.7–83.8)	NE	NE	NE	NE	NE
HPV-16, -18, -6, -11	**4.9 (1.4–16.4)**	1.9 (0.2–15.7)	2.3 (0.5–10.6)	NE	0.7 (0.1–5.7)	1.9 (0.2–15.7)	3.0 (0.6–15.0)	**5.3 (2.0–14.0)**

NOTE. NE, OR not estimable because there were no cases with the specific combinations.

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It is of interest to note that the increased risk of concurrent and sequential HPV infection that we observed in our cohort was not restricted to HPV types within the same phylogenetic group. However, our data also suggested a trend toward more-frequent subsequent coinfections with HPVs from a different phylogenetic group, which could be a possible manifestation of some degree of cross-protection between viruses with genetic similarity.

Our study differs in several respects from previous studies of HPV coinfection. It included a large cohort of middle-aged women from a population at high risk for HPV infection and cervical cancer. Only 1 of the 3 previous studies, which was conducted in Brazil, had a comparable study population [7], but the other 2 included only young women [5, 6]. In addition, our follow-up period was longer—we included in the present study 6 consecutive visits, with a median of 7 months between them. Importantly, we included in the analysis only incident infections in subjects with normal cytological results, and we excluded prevalent HPV infections for which we could not ascertain the date when subjects became infected. A fraction of the prevalent infections could have been persistent, and this makes difficult the separation of coinfection effects from persistence effects.

We estimated the number of expected counts of multiple infections by assuming a Poisson distribution. This analysis validated the estimation of expected counts by simulation, as was reported earlier by Thomas et al. [6]. Using the observed marginal probability of each HPV type for equal conditions of the actual data (i.e., using the same number of visits per person and excluding baseline prevalent visits) and assuming that specific HPV type probabilities were independent, we estimated the expected number of single and multiple infections, repeating the simulation process 1000 times. The expected values that we found (data not shown) were equivalent to those obtained under the assumption of a Poisson distribution.

In addition, the Poisson model was expanded to a negative binomial distribution that corresponds to a gamma Poisson hierarchical model, to quantify how the probability of infection with multiple HPV types increased across the data (i.e., clustering). The estimates of clustering represent variance-to-mean relative changes.

We also replicated the statistical procedures followed by Thomas et al. [6], which evaluated concurrent and subsequent infections for 6 HPV types. In our study, estimates of observed and expected counts of concurrent infections were performed for 36 different HPV types. In addition, the analysis of association was expanded by use of pairs of visits as outcome units and by use of robust regression methods to adjust for the correlation of repeated measures. The main advantage of using pairs of visits instead of subjects as units of analysis is that it incorporates the time-varying nature of the exposures to different HPV types.

One potential limitation of our study is that, despite our use of PCR assays of high sensitivity, we may have missed cases of infection in which the viral load was lower than the threshold of HPV DNA detection; this may have led to the misclassification of some concurrent persistent infections as subsequent new infections. Moreover, the $GP5^+/GP6^+$ system that we used is less sensitive for detecting multiple infections than other systems, such as the MY09/11 assay [15]. Another limitation of our study refers to the definition of incident HPV; we cannot rule out that what we called incident infections were, in fact, reactivations of latent infections. In addition, a limitation could be that, when pairs of visits are used as analysis units, it may be difficult to disentangle the effect of concurrent from that of subsequent infections. However, it is reassuring that our results are in agreement with those of previous studies in which women were used as units of analysis [5, 6, 7].

In conclusion, the increased risk of both concurrent and sequential HPV infections observed in our cohort suggests a common mode of transmission or special host susceptibility that predisposes some women to infection with certain HPV types or a potential beneficial impact of HPV vaccines on the prevalence of other types. This might represent an added value to prophylactic vaccines that are capable of preventing HPV infection and cervical intraepithelial neoplasia [16, 17].

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