Research Article

Concurrent Infection with Multiple Human Papillomavirus Types: Pooled Analysis of the IARC HPV Prevalence Surveys

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Abstract

To understand viral interactions and the cross-reactivity of natural or vaccine-induced responses, we investigated whether multiple human papillomavirus (HPV) infections, particularly certain combinations of types, have the tendency to cluster together. Cervical cell samples were collected from women in the framework of the IARC HPV Prevalence Surveys. Women with a cytology diagnosis of high-grade squamous intraepithelial lesion or worse were excluded, leaving 13,961 women for this analysis. HPV DNA was assessed using a general GP5+/6+ primer–mediated PCR. HPV genotyping was done using enzyme immunoassay or reverse line blot analysis. Logistic regression with type-specific HPV positivity as an outcome was used, adjusted for age, study area, and lifetime number of sexual partners. Woman-level random effects were added to represent unobservable risk factors common to all HPV types. The observed-to-expected ratio was 1.20 (95% credible interval, 1.14-1.26) for infection with two HPV types and 1.02 (95% credible interval, 0.91-1.12) for three for more types, with the best possible adjustment. Among combinations of specific HPV types, the tendency to cluster increased with the genetic similarity of the L1 region. High observed-to-expected ratios were found for closely homologous types, including HPV33/58, 18/45, 33/35, and 31/35. The excess of multiple infections, however, was clearly evident only when enzyme immunoassay, and not reverse line blot, was used as the genotyping method. The different results by genotyping method suggest that the apparent clustering of HPV infections was an artifact of the measurement process. Further investigation is required to evaluate other widely used HPV detection methods. *Cancer Epidemiol Biomarkers Prev; 19(2); 503–10. ©2010 AACR.*

Introduction

Genital infection with human papillomavirus (HPV) is a common sexually transmitted condition (1, 2) and is a necessary cause for cervical cancer (3). More than 40 anogenital HPV types exist, of which 12 have been classified as carcinogenic to humans (4). Coinfection with multiple HPV types is found in 20% to 50% of HPV-infected women (5, 6).

The implications of infection with multiple HPV types remain unclear. Although the tendency of multiple HPV types to cluster within women has been frequently observed (5, 7-11), follow-up studies suggest that the presence of multiple types does not influence the course of HPV infections (12, 13). In addition, the antibody levels elicited by natural HPV infection are low (14-16).

In view of these findings, the excess of multiple HPV infections has generally been explained by the shared transmission route and by the association of all HPV types with the same risk factors (7, 9), that is lifetime number of sexual partners, husband's sexual behavior (1, 2), and recent sexual partners (17).

However, the interpretation of previous studies has been hampered by limitations in sample size, together with the difficulty of accounting sufficiently well for risk factors common to all types. The aim of the present study is to give an in-depth evaluation of the prevalence of multiple infections and of the clustering pattern of the most common HPV types.

Materials and Methods

Contributing Studies and Data Collection

A series of population-based HPV Prevalence Surveys has been carried out by IARC in 15 areas in four

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continents between 1993 and 2007, using a common protocol. The surveys were carried out in the following areas: Hanoi and Ho Chi Minh City, Vietnam (18); Lampang and Songkla, Thailand (19); Korea (20); Shanxi (21), Shenzhen (22), and Shenyang (23), China; Mongolia (24); Argentina (25); Chile (26); Colombia (27); Nigeria (28); Spain (29); and Poland (30). A survey from Dindigul Distreet, India (31) was not included because information on sexual behavior was inadequate. Briefly, in each area, an attempt was made to obtain a random age-stratified sample of the population that included at least 100 women in each 5-year age group, from 15 to 19 y, to 65 y and over. Participation ranged from 48% in Songkla, Thailand (where most nonparticipants were not found at the address given by the population list), to 96% in Colombia.

Exclusion criteria were pregnancy at time of recruitment, previous hysterectomy, and physical or mental incompetence. Trained interviewers questioned study participants face-to-face using a questionnaire that included information on sexual behavior (2). All participants signed informed consent forms according to the recommendations of the IARC and the local ethical review committees, which approved the study.

Gynecologic Examination, Specimen Collection, and Cytology

Study participants underwent a vaginal examination during which samples of exfoliated cells from the ectocervix were collected. These were placed in tubes with PBS and were stored on ice. Cells were centrifuged at 3,000 g and the resulting pellets were resuspended, in the earliest studies, in PBS and then frozen between −20°C and −80°C until they were shipped to IARC for storage. Cervical cells were stored in cytoRich (Tripath Imaging) medium in the three studies from China, and in PreservCyt (Hologic, Inc.) medium in those from Mongolia and Poland. Conventional or liquid-based cytology smears were read locally and classified according to the 2001 Bethesda system.

HPV DNA Detection Techniques

HPV testing was done on exfoliated cervical cells in the Department of Pathology, Vrije University Medical Center, Amsterdam, the Netherlands. Only women who tested positive for β-globin, which is used as a marker for DNA quality, were included in this analysis.

A first screening was done to determine the overall presence of HPV DNA using a general GP5+/6+ primer–mediated PCR (32). HPV positivity was assessed by hybridization of PCR products in an enzyme immunoassay using oligoprobe cocktails to detect the following 36 HPV types: HPV6, 11, 16, 18, 26, 31, 33-35, 39, 40, 42 to 45, 51, 52 to 59, 61, 66, 68, 70, 71 (equivalent to CP8061), 72, 73 (equivalent to MM9), 81 (equivalent to CP8304), 82 (IS39 and MM4 subtypes), 83 (equivalent to MM7), 84 (equivalent to MM8), and CP6108 (32).

Subsequently, GP5+/6+ PCR products were used for HPV genotyping by one of two methods: (a) enzyme immunoassay (EIA; ref. 32) following hybridization with type-specific 30-mer oligoprobes at 37°C or (b) reverse line blot analysis (RLB) involving hybridization with type-specific 17- to 21-mer oligoprobes at 42°C (33). Method a was used in the older HPV Surveys, that is Hanoi and Ho Chi Minh City in Vietnam, Lampang and Songkla in Thailand, Argentina, Colombia, Nigeria, and Spain, whereas method b was used in the most recent HPV Surveys, that is Korea, Shanxi, Shenzhen and Shenyang in China, Mongolia, Chile, and Poland.

EIA Genotyping

The EIA typing procedure was done as previously described by Jacobs et al. (32) using individual, HPV type-specific digoxigenin-labeled 30-mer oligonucleotide probes (Eurogentec). Oligonucleotide sequences were presented before (32, 34). Briefly, PCR products were captured in streptavidin-coated wells of a microtiter plate (Roche; 5 µL per well for each type) for 60 min at 37°C, denatured by alkaline (0.2 N NaOH) treatment for 15 min at room temperature, and hybridized to the labeled oligonucleotides for 60 min at 37°C. The resulting hybrids were detected after incubation with antidigoxigenin-conjugated (Fab fragments) alkaline phosphatase (Roche) for 60 min at 37°C, followed by p-nitrophenyl phosphate substrate incubation overnight at 37°C. Finally, absorbance was read with an EIA reader at 405 nm using a reference filter at 620 nm.

RLB Genotyping

RLB genotyping was done with the aid of a miniblotter to apply HPV type–specific oligonucleotide probes, about 17 to 21 nucleotides in length and provided with a 5′ terminal NH_2 group (Isogen), to an activated negatively charged membrane as previously described (33, 35). The oligonucleotides were applied in parallel lines, and after a 1-min incubation, the channels were aspirated and the membrane was inactivated using 100 mmol/L NaOH. For hybridization, 10 µL of PCR product were diluted in 150 µL of 2× saline-sodium phosphate-EDTA/0.1% SDS, heat-denatured (96°C), and rapidly cooled on ice. The PCR products were pipetted into the parallel channels perpendicular to the rows of oligoprobes. Hybridization was done at 42°C for 1 h, followed by two washings in 2× saline-sodium phosphate-EDTA/0.5% SDS at 51°C. Subsequently, membranes were incubated with streptavidin-peroxidase conjugate for 45 to 60 min at 42°C. Detection was done, by using enhanced chemi-luminescence detection liquid (Amersham) followed by an exposure to a film (Hyperfilm) for 1 to 10 min. Films were subsequently developed.

Sequence Alignment and Phylogenetic Analysis

Prototype sequences for specific HPV types were obtained from the National Center for Biotechnology Information/Genbank database. Subsequently, the DNA sequence data of L1 genes were aligned with ClustalW2 using default options (36, 37). The similarity between

HPV types was measured by the percent identity scores in the best sequence alignment.

Statistical Analyses

The analysis of clustering of HPV types was restricted to HPV types with a prevalence of at least 0.5% in the whole study population. HPV types with lower prevalence were considered too rare to provide information on clustering in multiple infections. Fifteen types were thus considered, including 11 carcinogenic types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, and 58) and 4 other types (HPV42, 66, 70, and 81; refs. 4, 38).

Multivariate logistic regression was used to model type-specific HPV positivity. The presence of each HPV type was considered as a separate outcome for each woman. Covariates in the model were age (<25, 25-34, 35-44, 45-54, ≥55 y), study area, and lifetime number of sexual partners, as indicated.

Because the data have a hierarchical structure, with HPV infections nested within women, multilevel models were used, with woman-level random effects. In this context, a random effect is an unobserved quantity that varies between women. Random effects account for the fact that women with the same observable risk factors may have different levels of risk for prevalent HPV infection and that type-specific HPV measurements in the same woman are correlated with each other. Woman-level random effects represent all sources of residual variation in the risk of HPV infection other than those already represented by the covariates, that is, unobserved host or environmental risk factors, and are particularly useful to overcome the limitations of questionnaire variables (that is, lack of availability and relatively poor quality of certain variables) as predictors of HPV positivity.

Interaction terms for study area by age and study area by HPV type were included in the model to account for our previous findings that the age profile of HPV infection (39) and the ranking of different HPV types by prevalence (6) vary between study areas. These interaction terms were also modeled as random effects.

Models were fitted using a Bayesian approach, using Markov Chain Monte Carlo simulation. Estimates are reported as posterior means and 95% credible intervals (95% CI). This method was chosen because it allows interval estimates to be calculated for arbitrarily complex summaries of the model parameters. Compatible results were obtained when the models were fitted with MLWin (40).

Discrepancies between the data and the model were measured using observed-to-expected (O/E) ratios for the counts of multiple infections. Goodness of fit of the model was assessed by posterior predictive two-sided P values (41), with O/E ratios as a test statistic. Because all possible two-way interactions between the 15 HPV types were tested, this generated 105 P values. When testing 105 different hypotheses, one would expect approximately five falsely positive associations due to chance with a significance level of 0.05, and approximately one with a significance level of 0.01. Therefore, to minimize errors arising from multiple comparisons, the threshold for significance was set to 0.01 instead of 0.05.

Results

Of 14,536 women recruited into the 15 studies, 14,176 had valid cytology and HPV results, and information on lifetime number of sexual partners. Cytologic abnormalities (defined as atypical squamous cells of undetermined significance or worse) were found in 907 (6.4%) women, with prevalence varying between study areas from 0.7% in Hanoi, Vietnam, to 20.4% in Shanxi, China. Two hundred and fifteen women with a cytology diagnosis of high-grade squamous intraepithelial lesion or worse were excluded, leaving 13,961 women.

The mean age was 40.8 years, varying between 32.7 years in Colombia and 47.8 years in Songkla, Thailand. Overall, HPV prevalence was 12.3%, ranging between 1.3% in Hanoi, Vietnam and 28.9% in Mongolia (Fig. 1A). Multiple infections were found in 32.2% of HPV-positive women, ranging from 18.5% in Korea to 46.0% in Argentina.

Figure 1B shows the proportion of individual HPV types found in single and multiple infections. HPV16 was the carcinogenic type that was most often (58.5%) found alone, whereas HPV33, 35, and 45 were detected in over 70% of infected women in combination with other types.

Table 1 shows the observed and expected numbers of women with single and multiple HPV infections (one type, two types, and three or more types) under three different statistical models of increasing complexity. The basic model included only age, study area, and HPV type as covariates. For this model, the O/E ratio was 1.66 (95% CI, 1.52-1.80) for infection with two HPV types and 7.25 (95% CI, 6.04-8.65) for infection with three or more HPV types. The adjusted model included additionally a woman's lifetime number of sexual partners (1, ≥2) as a covariate. With this model, the O/E ratio was 1.62 (95% CI, 1.49-1.74) for two HPV types and 6.43 (95% CI, 5.31-7.62) for three or more HPV types. Inclusion of the number of sexual partners in the model thus reduced the O/E ratio slightly. The full model included random intercepts for individual women, representing unobserved host or environmental risk factors for HPV infection that are common to all types. With the full model, the O/E ratio for infections with two types was 1.20 (95% CI, 1.14-1.26) and 1.02 (95% CI, 0.91-1.12) for infections with three or more types. The discrepancy between observed and expected counts was thus attenuated substantially, although there was still a small excess of infections with two types. The existence of this excess motivated an investigation into specific interactions between HPV types.

Figure 2 shows P values for tests of the hypothesis of no association between two HPV types, under the full
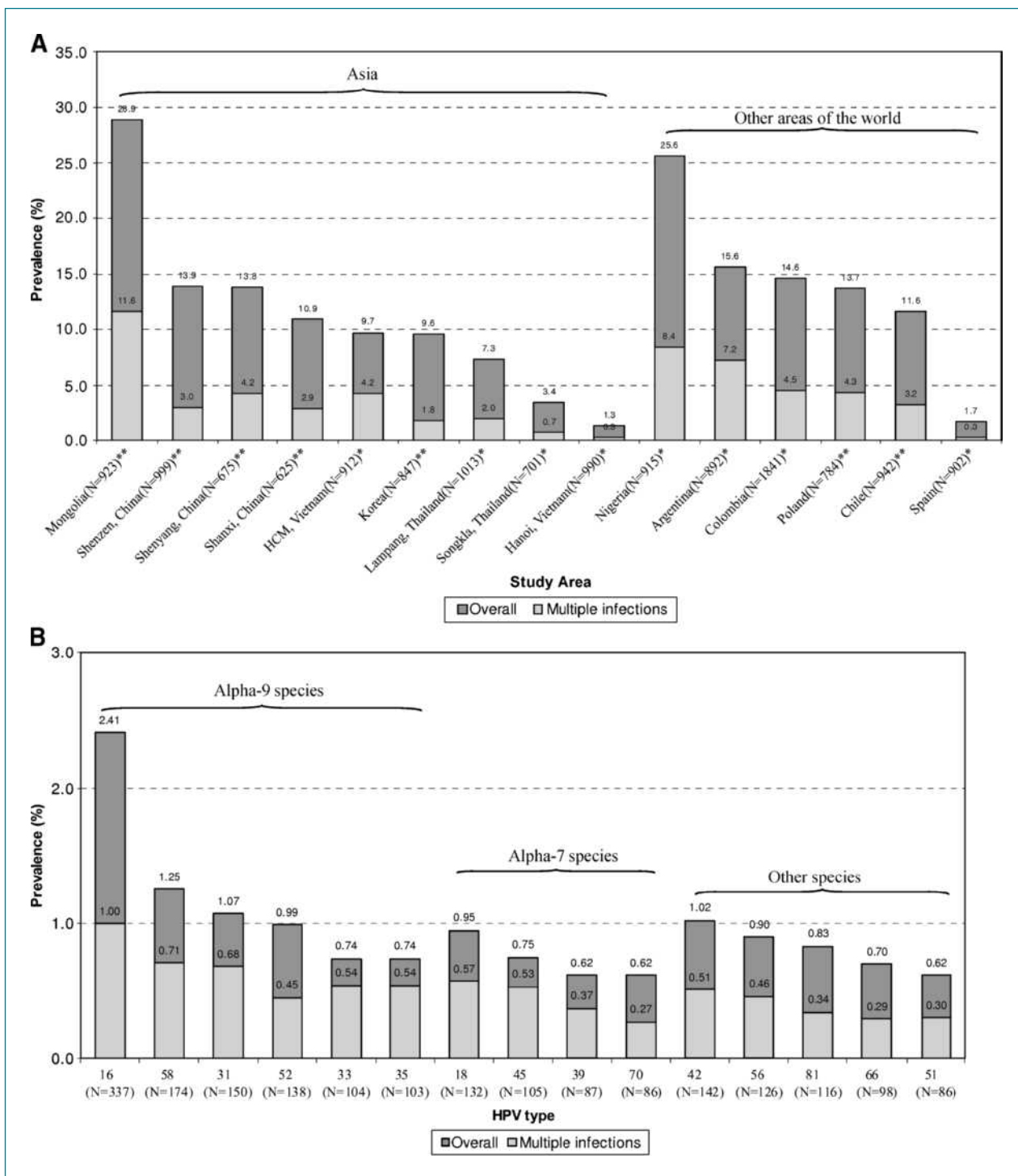
Figure 1. A, prevalence of any HPV type among 13,961 women, by study area. B, type-specific HPV prevalence in 13,961 women. *, EIA typing. **, RLB typing.

model. Separate *P* values are presented for each two-way combination of the 15 most common types. A *P* value of <0.01, was observed for six pairs of HPV types. Five of these six HPV pairs (HPV33/35, 33/58, 33/39, 18/45,

and 31/35) were found significantly more often that expected and one pair (HPV16/81) was found significantly less often than expected. According to the phylogenetic classification of de Villiers et al. (42), four of the five pairs

Table 1. O/E ratio of multiple infections with the 15 most common HPV types, according to various models

No of HPV types		Basic model		Adjusted model		Full model	
	O	E*	O/E (95% CI)*	E†	O/E (95% CI)†	E‡	O/E (95% CI)‡
0	12,510	12,170.8	1.03 (1.02-1.03)	12,183.0	1.03 (1.02-1.03)	12,496.1	1.00 (1.00-1.01)
1	1,067	1,610.5	0.66 (0.64-0.69)	1,592.4	0.67 (0.65-0.69)	1,127.6	0.95 (0.91-0.99)
2	271	163.9	1.66 (1.52-1.80)	167.9	1.62 (1.49-1.74)	226.2	1.20 (1.14-1.26)
3-7	113	15.7	7.25 (6.04-8.65)	17.7	6.43 (5.31-7.62)	111.1	1.02 (0.91-1.12)

Abbreviations: O, observed; E, expected.
*Controlling for age and study area, and interactions for study area–age and study area–specific HPV type.
†As * plus lifetime number of sexual partners.
‡As † plus individual random effects.

that occurred together more often than expected are from the same α species. Conversely, in the single pair of HPV types that were found less often than expected, the two types were from different α species. Supplementary Table S1 shows more details for these six pairs.

Further results on the tendency of closely related HPV types to cluster together are given in Table 2, which shows O/E ratios of coinfections with HPV pairs stratified by the percent identity of the DNA sequences in the L1 region. The majority of the HPV pairs ($n = 79$) showed a percent identity between 59% and 69%, whereas 22 pairs had a percent identity score included between 70% and 79% and four pairs 80% or above.

Different results were found for EIA and RLB genotyping methods, so the findings are presented separately.

Among 8,166 women in the older IARC HPV Prevalence Surveys that used EIA for typing, the O/E ratio was 0.74 (99% CI, 0.60-0.95) for pairs with a percent identity of <70%, 1.23 (99% CI, 1.00-1.55) for pairs with a percent identity between 70% and 79%, and 3.03 (99% CI, 2.28-4.01) for pairs with a percent identity ≥80%. The formal test for linear trend of O/E ratios was highly significant ($P = 0.0003$). Conversely, among 5,795 women from more recent HPV Surveys that used RLB, the O/E ratio was 0.82 (99% CI, 0.68-1.01) for pairs with a percent identity of <70%, 0.84 (99% CI, 0.70-1.07) for pairs with a percent identity between 70% and 79%, and 1.25 (99% CI, 0.99-1.60) for pairs with a percent identity of ≥80%. The test for trend of O/E ratios was not significant ($P = 0.47$).

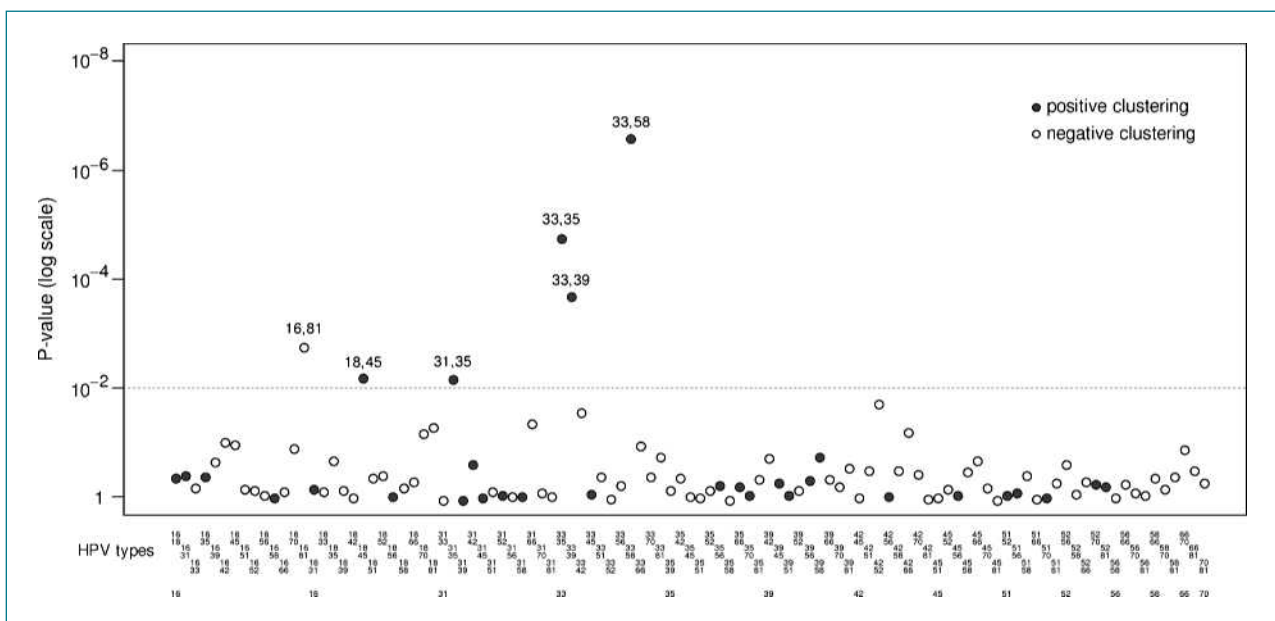Figure 2. *P* values for independence of joint HPV infections, as estimated by the full model.

Table 2. O/E ratio of coinfections with HPV pairs, aggregated according to the percent identity of DNA of the *L1* gene, stratified by typing procedure

Percent identity, %	No. of pairs	EIA			RLB		
		No. of coinfections with HPV pairs			No. of coinfections with HPV pairs		
		O	E	O/E* (99% CI)	O	E	O/E* (99% CI)
59-69	79	198	266.5	0.74 (0.60-0.95)	249	302.8	0.82 (0.68-1.01)
70-79	22	137	111.2	1.23 (1.00-1.55)	97	114.8	0.84 (0.70-1.07)
80-100	4	35	11.6	3.03 (2.28-4.01)	16	12.8	1.25 (0.99-1.60)
Linear trend test				*P* = 0.0003			*P* = 0.47

Abbreviations: O, observed; E, expected.
*As estimated by the full model.

Discussion

In our large HPV surveys that used GP5+/GP6+ PCR assays as the HPV detection method, we found that multiple HPV infections occurred more often than would be expected by chance, even after controlling for all sources of correlation between HPV types, and we observed that the tendency of infections to cluster increased with the genetic similarity of the specific HPV types. The excess of multiple infections, however, was clearly evident only when EIA, and not RLB, was used as a genotyping method.

Clustering of HPV types within women has been observed in previous studies with both cross-sectional and longitudinal designs and using different primers for HPV detection, including MY09/11 (5, 7, 8, 10) and GP5+/6+ (9) primers. Few studies, however, have been able to evaluate two-type clustering across a range of individual HPV types. Thomas et al. (7) evaluated HPV6, 11, 16, 18, 31, and 45 among 518 female university students in the United States using MY09/11 primers. No two-type infections were more or less likely than any other combination. Chaturvedi et al. (10) also used the MY09/11 system and focused on the clustering of 27 HPV types in multiple infections among 854 HIV-negative and 275 HIV-positive women from the United States, but they used α species rather than individual HPV types as the unit of their analyses. In a study of 1,610 Colombian women who were tested using GP5+/6+ with subsequent EIA genotyping method, as in our present study, Mendez et al. (9) reported an excess of clustering for several two-type combinations (including HPV33/58, HPV33/39, and HPV18/45, as we did), after adjustment for age and lifetime sexual partners. No data were shown on HPV35.

Previous studies were based on small numbers compared with our present study, which included ~14,000 women, of whom 1,720 were HPV-positive and 554 had multiple HPV infections. This large multicentric study,

using a common protocol in all centers, allowed a systematic investigation of multiple infections with specific two-type combinations among the 15 most common HPV types. The use of a statistical model with mixed effects allowed us to account for, in addition to the effects of measurable risk factors, the correlation between HPV types, due to unobserved risk factors common to all HPV infections. This means that when interpreting the excess of multiple infections between certain HPV types, we should rule out residual confounding due to any risk factor, observed or unobserved, common to all HPV types. After accounting for these common risk factors, there could be other explanations for the observed excess of multiple HPV infections, including the possibility of a biological interaction between certain two-type combinations. However, the different results by genotyping method strongly suggest that the apparent clustering of HPV types is an artifact of the genotyping methods and HPV types are independent infections. Cross-hybridization might occur when the DNA probe designed to match a specific DNA sequence hybridizes with homologous sequences from another HPV type, leading to the apparent detection of two HPV types when only one is present.

Our results showed that when EIA typing was used, HPV33/58, together with 18/45, 33/35, and 31/35, were among the HPV pairs that contributed the most to the apparent excess of multiple infections. The two HPV types in each of these pairs shared a substantial portion of the ~1,500 bp of the *L1* gene, with highest values being found for HPV33 with 58 (85 percent identity) and for HPV18 with 45 (81 percent identity). The probes used by the EIA genotyping method also had a high similarity for some of these HPV pairs. For each two-way combination of HPV types, we aligned the 30 bp corresponding to the probe sequences, finding highest homologies for HPV33 with 58 probe (76 percent identity) and for HPV18 with 45 probe (52 percent identity). The similarity between the probe regions of the HPV types, might, together with the probe lengths and hybridization

temperature, have favored the occurrence of cross-hybridization. By comparison, RLB genotyping uses shorter probes (17-21 bases) at a higher hybridization temperature (42°C), ensuring much higher stringency conditions.

Our findings confirm the earlier suspicion of cross-hybridization reported by van den Brule (33) under the previously used conditions of EIA at a hybridization temperature of 37°C and also reveal a general tendency of all closely related HPV types to be detected together in studies using this EIA typing system. The much weaker aggregation among closely related HPV types observed in studies using RLB typing also shows the improved specificity of the method.

In conclusion, to clarify whether certain combinations of HPV types are more common than expected by chance, it is important to better understand viral interactions and the cross-reactivity of natural or vaccine-induced responses. Different primers for HPV detection can differ remarkably in their sensitivity to detect multiple infections (43-45). We showed, however, that the genotyping methods can also substantially affect results and lead to systematic overestimates of certain HPV combinations. The fact that the estimates of HPV prevalence for certain specific HPV types are not measured precisely has important implications for clinical studies of HPV persistence, as well as evaluations of cross-protection of HPV vaccines. Although great im-provements have been made already in PCR-based geno-typing, further investigation is required to evaluate other currently widely used HPV detection methods.

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