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Multiple-type HPV infections: a cross-sectional analysis of the prevalence of specific types in 309,000 women referred for HPV testing at the time of cervical cytology

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Abstract

Objectives—To determine the frequency of multiple type cervical HPV infections, and whether any types are involved in multiple type infections more or less frequently than might be expected if these infections occur randomly.

Methods—In this retrospective analysis of type-specific HPV testing, results from women 18-65 years old with samples collected between July 2007 and May 2011 were considered. Multivariate logistic regression analysis was used to model the presence of each of the 24 most prevalent HPV types, adjusting for one other HPV type, age, laboratory region, and age by region interactions.

Results—HPV infection was present in 74,543 of 309,471 women (24.1%) and 65,492 (21.1%) were positive for one of the top 24 most prevalent HPV types. The most common HPV type was HPV type 16, occurring in 4.1 of the entire sample%. 14,181 women were positive for 2 or more HPV types (4.6% of entire sample, 19.0% of HPV positive sample). Two-way HPV type comparisons were analyzed. Types 52, 53, 81, and 83 more likely to occur in multiple infections with other types, and types 16, 58, and 66 were less likely to occur in multiple infections with other types. HPV types 72 and 81 have the strongest positive relationship (OR=5.2, 95% CI: 3.6, 7.4). HPV types 33 and 66 have the strongest negative relationship (OR 0.4, 95% CI: 0.2, 0.6).

Conclusions—In this population, multiple type HPV infections were present in 4.6% of all women. Our findings suggest that there may be both competitive and cooperative interactions between HPV types.

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Keywords

DNA Probes; HPV; Epidemiology

Introduction

Human Papillomavirus (HPV) infection has been identified as the etiology cause of cervical carcinoma [1]. Over 100 different HPV types have been identified to date, and several have been identified as the principal cause of invasive cervical cancer and precancerous lesions. HPV infection remains the most common sexually transmitted disease [1].

While most HPV infections are characterized by spontaneous viral clearance, which usually occurs within 1-2 years, there are some infections which are persistent in nature. We have shown that 39% of women who had consecutive HPV testing as a component of cervical cancer screening had persistent HPV infection, of which 34% were high risk HPV types [2]. Persistent high risk HPV infection is an important predictor for the development of CIN 2/3 and invasive cervical cancer [3].

Multiple-type HPV infections have been documented in several epidemiologic studies. Chaturvedi et al have published several studies documenting as high as 43.2% of the Costa Rica HPV Vaccine Trial's population having multiple type infections [4]. While the observed frequency of multiple type infections in this population was more common than expected by chance, there was no evidence of specific HPV genotype interactions. Carozzi et al, in conjunction with the New Technologies for Cervical Cancer Screening (NTCC) working group, have reported an excess of multiple infections, though small, after controlling for sources of common correlation between HPV types [5]. In a study from Rousseau et al of 2075 Brazilian women, co-infection of HPV types were analyzed and compared to cervical cytology [6]. In this large cohort, infection with HPV types 16 and 18 were less likely to occur with other oncogenic HPV types. There continues to be a need for more studies evaluating the incidence of multiple type HPV infections, and any interaction between HPV types within these infections. These interactions can be imperative for understanding the changing milieu of HPV infection over time in this era of vaccination and concern for HPV type replacement. Our objectives were to determine the frequency of multiple type infection in a large cohort of women, and determine if any HPV types are identified in multiple-type infections more or less frequently than might be expected than by chance alone. While the incidence of multiple-type HPV infection has been reported, it is unknown whether HPV types co-infect in a random fashion. With the increasing administration of the HPV prophylactic vaccines, the mechanism behind multiple type infections warrants further investigation, in light of concern for HPV type replacement. To address this, we determined the frequency of multiple-type infection with high risk HPV types in a large population of women referred for HPV testing as a component of cervical cancer screening in the United States. The interaction between different phylogenetic species was also analyzed to assess if there are biological interactions between species that contribute to multiple-type infections.

Materials and Methods

Study Population

Prior to the initiation of this investigation, appropriate approval was granted by the University of Minnesota's Institutional Review Board. We performed an analysis of data from women who had HPV typing performed by Access Genetics (Eden Prairie, MN) between July 2007 and May 2011. Access Genetics offers medical diagnostic services,

including HPV testing. In addition to reporting the presence or absence of high-risk HPV types, they perform PCR-based HPV typing. Data from 47 labs which use Access group for HPV typing were analyzed. Most frequently, HPV typing was performed after an abnormal pap test or an abnormal colposcopy [2]. Patient age at testing, laboratory location, and test media type were the only demographic information available.

Specimen Analysis

Samples were processed within 48 hours of being received in liquid cytology medium. DNA was extracted by salt precipitation using a reagent from the commercial product PureGene (Gentra Systems, Minneapolis MN). Pap tests that had been collected in Thin Prep (Hologic Inc, Marlborough MA) solution (41.6% of samples) were processed from 1ml of cell/liquid mixture then centrifuged into a cell pellet. Pap tests that had been collected in the SurePath (BD Diagnostics, Burlington, NC) medium (46.7% of samples) were processed from residual cell pellets. In both cases, cells were then washed with 4ml of phosphate buffered saline (PBS), vortexed, and centrifuged at 2000g for 10 minutes to a pellet of cellular material. The supernatant was then decanted and 1ml of PBS was added to each sample, followed by transfer to 1.5ml microcentrifuge tubes. These tubes were then centrifuged at 4350 rpm for 5 minutes. The cellular fraction was then washed with PBS, vortexed, and again centrifuged to a pellet of varying volume. A volume of cell lysis and Proteinase K was added to each tube on the basis of visual inspection of the cell pellet volume, compared with a standardized template. Cell lysis was achieved and the mixture was incubated overnight at 55°C.

Following cell lysis, Protein Precipitation Solution (1/3 volume of cell lysis; Pure-Gene) was added. The sample tubes were incubated in an ice slurry for 5 minutes, followed by centrifugation at 7500 rpm for 10 minutes. The resulting supernatant was transferred to a clean 1.5ml microcentrifuge tube. DNA precipitation was achieved by adding isopropanol followed by repeat centrifugation at 7500 rpm for 10 minutes. The DNA was then washed in 70% ethanol, vortexed to disaggregate the pellet, and then the DNA was re-pelleted. The ethanol was decanted, and the pellet was then dried at 55°C for 20 minutes. This ethanol wash was performed until the pellet was clear. The dried DNA samples were then reconstituted with DNA hydration solution by PureGene at 55°C for 3 hours or overnight at room temperature. The amount of DNA hydration fluid was determined by the DNA volume that was present during extraction.

PCR amplification

Amplification of the genomic DNA was achieved by using modification of the methods of Resnick et al. [6]. Two independent reactions were assembled. Reaction A contained a combination of HPV specific primers; AG con forward (CGTCCMARRGGAWACTGATC), AG con reverse (GCMCAGGGWCATAAAYAATGG) (where the designation of M=A&C, R=A&G, W=A&T, and Y=C&T combinations of nucleotides) and 0.04 pmol PCO4 (CAACTTCATCCACGTTCCACC) specific for the human beta-globin gene. Reaction B contained the HPV primers only. Both sets of reactions were constructed from a master mix containing: 50 µL with 1.25 units of *Taq* polymerase (Promega, Madison WI), 10 mM Tris HCl, pH 8.3, 4.0 mM MgCl₂, 50 mM KCl (Buffer A), 0.2 mM of each dNTP, 8.0 pmol of each primer and 2-5 ng of genomic DNA. The reactions were cycled at 95 °C for two minutes followed by 95°C for 20 seconds, 55°C for 20 seconds, and 72°C for 30 seconds. The final cycle was extended to 72°C for five minutes and then held at 4-15°C until analyzed.

The products of PCR amplification were analyzed by methods of polyacrylamide gel electrophoresis. 8µL each of reaction A and B were combined with 3µL of 5× loading

mixture. Samples were mixed in a series of striptubes, loaded onto gels, and electrophoresed in 1× Tris Borate EDTA for one hour at 120 volts. The separated DNA products were stained with ethidium bromide, illuminated under ultraviolet light, and the image was captured digitally (UVP System, Worcester MA). The expected size of the HPV amplicon is 415-464 bp and it is 260 bp for beta-globin.

Next, samples positive for HPV were subjected to genotyping by restriction endonuclease fragment analysis. Briefly, 10µL of the reaction B product was mixed with a solution containing 10µL of a master mix composed of one of the restriction endonucleases Pst I, Rsa I, or Hae III (1U) in their respective buffers. Each enzyme reaction was digested individually for two hours at 37°C. Following digestion, the resulting fragments were analyzed by polyacrylamide gel electrophoresis using pre-cast 6% 18-lane gels (Biorad, Hercules CA).

Interpretation of the HPV genotypes was based on the pattern of resulting bands for each enzyme, which was compared to the genomic maps of each viral type. The pattern of restricted DNA bands for each of the known HPV viral types has been described previously [7]. The 22 types considered high-risk were HPV-16, -18, -26, -31, -33, -35, -39, -45, -51, -52, -53, -56, -58, -59, -66, -67, -68, -69, -70(LVX160), -73(MM9), -82(MM4, IS39), and -85. Low-risk HPV types in this study were HPV-6, -11, -32, -40, -42, -44, -54, -55, -61, -62, -64, -71(CP8061), -72(CP4173, LVX100), -74, -81(CP8304), -83, -84(MM8), -87, -89(CP6108), and -91 [8]. The subtypes listed in parentheses were combined with their primary type for analysis. All of the other detected HPV types were categorized as unclassified (unknown risk).

Statistical Methods

The first adequate samples for HPV type evaluation from each woman collected between July 2007 and May 2011 were considered (n=325,486). When limiting to those who were 18-65 years old at testing, a total of 309,471 women remained. The only demographic information available for all of these women was age at testing, laboratory location (Western, Southern, North Central, and Eastern United States) and test media type. All available patient demographic and clinical characteristics were summarized using descriptive statistics. The 24 most prevalent HPV types in the population were considered for analysis: 6, 11, 16, 18, 31, 33, 35, 45, 52, 53, 54, 58, 59, 61, 62, 66, 68, 70, 72, 81, 82, 83, 84, and 89. Prevalence of infection and multiple infections were calculated based on these types. Multivariate logistic regression was used to address the relationship between each pairing of HPV types. The positivity of each HPV type was considered as a separate outcome, with age, laboratory region, age by region interaction and positivity of one other HPV type as predictors in the analysis. Given the large sample size, the use of a full model, including the interaction of age and region, was decided a priori. No interactions with HPV type were considered, however. Whether a woman had more than 2 co-infections was not included in the model, however, all pairwise comparisons were considered. All women were included in all analyses.

HPV types were also analyzed according to their phylogenic viral species [9]. Prevalence of infection and multiple infections were calculated by virus specie. Multivariate logistic regression was performed between all possible pairs of phylogenic viral species using the same statistical methods as above.

The significance threshold was set at $p < 0.001$ in order to minimize errors arising from multiple comparisons. Odds ratios (OR) and 95% confidence intervals (CI) are presented. Statistical analyses were performed using SAS version 9.2 and R version 2.7.

Results

The mean age of these women at time of sample collection was 39.2 (SD = 12.1) years. The majority of samples sent to Access Genetics for HPV specific genotyping were from the East Coast of the United States (n=141,574; 45.8%).

Of those women where HPV type evaluation was performed, 74,543 (24.1%) were infected with at least one HPV type and 65,492 (21.1%) were positive for one of the 24 most prevalent HPV types. The most common HPV type was type 16, which occurred in 4.1% of all women, and as part of a multiple type infection in 1.2% of all women (Figure 1/Table 1). A total of 14,181 women were positive for 2 or more HPV types (4.6% of entire sample, 19.0% of HPV positive sample). Specifically, 12,016 (3.9%) were infected with two HPV types, 1,936 (0.6%) with three HPV types and 229 (0.1%) with four HPV types.

All two way HPV type comparisons were performed, and p-values are represented (Figure 2). 65 combinations were strongly significant ($p < 0.001$) and 38 were potentially significant ($p < 0.01$). As noted on Figure 2, those relationships which were more likely to be positively associated (more likely to occur together) are designated with green boxes, and those relationships more likely to be negatively associated (less likely to occur together) were noted in red. HPV types 52, 53, 81, and 83 were involved in multiple positive relationships, while HPV types 16, 58, and 66 were involved in all of the strongly significant negative relationships.

For those 65 combinations that were strongly significant, ORs and 95% CIs are presented (Figure 3). HPV types 72 and 81 had the strongest positive relationships (OR=5.2, 95% CI: 3.6, 7.4). HPV types 33 and 66 had the strongest negative relationships (OR=0.4, 95% CI: 0.2, 0.6).

The most common viral specie was α -9 (including types 16, 31, 33, 35, 52, 58, 67), with 9.6% of all women and 56.8% of women with multiple type infections being infected with one or more of these types (Figure 4/Table 2). The α -9 specie was also the only specie involved in negative relationships, where species α -3 (types 61, 62, 81, 83, 84, 89), α -5 (type 82), and α -6 (types 56, 66) were all involved in positive relationships (Figure 5).

When evaluating the incidence HPV infections based on high risk (HR-HPV) and low risk (LR-HPV) groups, 7.4% of all women had an infection with a LR-HPV type and 16.4% with a HR-HPV type. Of all multiple type HPV infections, 52.4% of all multiple type infections contain LR-HPV, and 84.3% contain HR-HPV.

Discussion

In the present study, we found that HPV was present in 74,543 of 309,471 (24.1%) women referred for testing as a component of cervical cancer screening. This prevalence is similar to other studies of large numbers of women who were tested for HPV at the time of cytology testing. Our data includes mostly women who were referred for abnormal cytology, and therefore may only be applicable to that population with abnormal cytology. In a study of 5,000 samples from the Centralized Cervical Cancer Screening Program of British Columbia, 16.8% were HPV positive, with 33% of that sample being positive for more than one HPV type [10]. In the ARTISTIC trial of 24,510 women, 27.3% of women under the age of 30 had high risk HPV types, while only 6.1% of women over the age of 30 had high risk HPV types at the time of cytology testing [11]. In our patient population, the mean age was 39.2, and therefore spans the two groups discussed in the ARTISTIC trial.

The Costa Rica HPV Vaccine Trial analyzed 5871 sexually active women and found a 50.0% prevalence of HPV [4]. Multiple type HPV infections were found in 18.2% of the women. In a longitudinal study of 2,113 Brazilian women followed for more than 10 years with serial cytology and HPV testing, Trottier et al. show that 1-year and 4-year cumulative prevalence of co-infection with multiple types was 12.3% and 22.3%, respectively [12]. In that same study, multiple low risk HPV types in a single infection exclusively did not increase the risk of HSIL. However, those patients infected with 2 or more high risk HPV types did have an increased risk of any grade SIL, even when excluding for persistent infections.

It is unknown whether the HPV types in multiple infections occur randomly, or if a competitive or cooperative relationship exists. The study of 2075 Brazilian women by Rousseau et al. had findings suggesting different HPV multiple-type infections were not independent of each other [6]. In an analysis of the Guanacaste Study of HPV Natural History, infections with multiple HPV types occurred more often than expected by chance; however, after controlling for various factors including lifetime number of sexual partners, age, and specific HPV type prevalence, the increased multiple type numbers were small [13]. They noted there was a tendency of HPV types to cluster with similarity of L1 regions in the genome. In the present study, we found that there were a total of 57 combinations that were strongly significant to be more likely to occur than expected and 8 combinations that were strongly significant to be less likely to occur than expected. This suggests that there may be some interaction between different types of HPV that is yet unknown. The study by Trottier et al also suggests an interaction between HPV types that can increase baseline risk of abnormal cytology [12]. This is similar to the study by Rousseau et al.

Using a similar population of women in Guanacaste, Costa Rica, a recent study of 8365 women documented that after controlling for sources of common correlation between HPV types, there was no evidence of a tendency to cluster positively or negatively in multiple infections [13]. However, a small degree of association between genetically related HPV types was documented.

We observed that HPV Types 72 and 81 have the strongest positive relationship, and types 33 and 66 have the strongest negative relationship. This illustrates that the relationship between the combinations is different for each HPV type and again suggests further research into the mechanism behind these relationships between HPV types is needed.

The International Agency for Research on Cancer (IARC) has described common ancestors for different HPV species [9]. We found that multiple-type infections involving the α -9 specie were most prevalent and the α -9 was the only specie involved in strongly negatively associated combinations, while all other families were involved in positively associated combinations. This indicates there may be a mechanism competing for co-infection involving α -9 types. This is supported by the study by Trottier et al. which hypothesizes HPV types in the α -9 specie could exert oncogenic effects and impact the cervical epithelia infected with other HPV types [12]. This observation is an important one in regards to the finding within the Costa Rica HPV Vaccine Trial's analysis of the α -9 specie and co-infection with multiple α -9 species was associated with significantly increased risk of CIN2 or HSIL, though this risk was similar to the sum of the estimated individual risks [1].

Because this was a retrospective study, we were limited by the data available to us. In particular, we were unable to link HPV testing with specific indications or cytology and histology, and do not have information on cytology for the entire population. We understand that this may mean our prevalence data of individual types may be skewed based on original cytology findings. As the majority of samples were sent for HPV testing based on abnormal

cytology, generalizations cannot be made to the population as a whole. Further studies linking cytology and histology are warranted to further assess the possible competitive and cooperative interactions between HPV types. However, the Costa Rica HPV Vaccine Trial did show that women with multiple infections were at significantly increased risk of CIN2 and HSIL when compared with those with single infections [4]. We were also unable to evaluate behavioral and treatment information of the women tested, as we did not have adjustment for sexual partners or other risk factors, such as smoking status. This as well is an important limitation of the study. In addition, PCR test results may have been affected by differences in sensitivity of the testing method for different HPV types.

Our large retrospective study suggests relationships between HPV types which may influence HPV epidemiology. A total of 57 two-HPV type combinations were found to be involved in positive relationships, and 8 two-HPV type combinations were found to be in negative relationships. The α -9 specie appears to be different than other species, as this specie was the only specie to have strongly negatively associated combinations when analyzing for specie type. Further investigation is needed to verify and to determine the mechanism of these potential relationships between types. If these hypothesized relationships exist, it will be important to consider them when understanding the impact of prophylactic vaccination on prevalence of various HPV types. Until these relationships are further understood, prophylactic vaccination cannot be individualized for each woman based on her HPV infections.

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Figure 1.
Type-specific HPV infection rates in 309,471 women in the United States.

Figure 2.
Associations between HPV types involved in Patients with Multiple-Type HPV Infections by Family.

Figure 3.
Odds ratios and 95% Confidence Intervals (CI) for all significant two-type HPV infections.

Figure 4.
Prevalence of Phylogenic specific HPV infection rates in 309,471 women in the United States.

Figure 5.
Phylogenic associations between HPV types Involved in Patients with Multiple-Type HPV infections.

Table 1

Prevalence of Type-specific HPV infection and 95% confidence intervals in 309,471 women in the United States.

HPV Type	Overall Prevalence (95% CI)	Prevalence -Multiple Infections (95% CI)
16	0.0412 (0.0405, 0.0419)	0.0118 (0.0114, 0.0122)
53	0.0264 (0.0259, 0.0270)	0.0095 (0.0091, 0.0098)
52	0.0206 (0.0201, 0.0211)	0.0076 (0.0073, 0.0079)
31	0.0194 (0.0189, 0.0199)	0.0061 (0.0058, 0.0064)
66	0.0188 (0.0183, 0.0192)	0.0065 (0.0062, 0.0068)
61	0.0142 (0.0137, 0.0146)	0.0050 (0.0048, 0.0053)
58	0.0114 (0.0111, 0.0118)	0.0036 (0.0034, 0.0039)
84	0.0101 (0.0097, 0.0104)	0.0040 (0.0038, 0.0042)
62	0.0096 (0.0093, 0.0099)	0.0038 (0.0036, 0.0040)
6	0.0095 (0.0092, 0.0099)	0.0036 (0.0034, 0.0038)
59	0.0083 (0.0080, 0.0087)	0.0038 (0.0036, 0.0040)
54	0.0079 (0.0076, 0.0082)	0.0032 (0.0030, 0.0034)
18	0.0072 (0.0069, 0.0075)	0.0026 (0.0024, 0.0027)
82	0.0071 (0.0068, 0.0074)	0.0029 (0.0027, 0.0031)
83	0.0068 (0.0066, 0.0071)	0.0024 (0.0023, 0.0026)
81	0.0054 (0.0051, 0.0056)	0.0024 (0.0022, 0.0026)
45	0.0049 (0.0047, 0.0052)	0.0018 (0.0016, 0.0019)
70	0.0044 (0.0042, 0.0047)	0.0014 (0.0013, 0.0015)
68	0.0041 (0.0039, 0.0043)	0.0015 (0.0014, 0.0017)
89	0.0040 (0.0038, 0.0042)	0.0016 (0.0014, 0.0017)
72	0.0039 (0.0037, 0.0042)	0.0010 (0.0009, 0.0011)
11	0.0036 (0.0034, 0.0038)	0.0012 (0.0010, 0.0013)
33	0.0036 (0.0034, 0.0038)	0.0010 (0.0009, 0.0011)
35	0.0030 (0.0028, 0.0032)	0.0010 (0.0009, 0.0011)

Table 2

Prevalence of Phylogenic-specific HPV infection and 95% confidence intervals in 309,471 women in the United States.

HPV Family	Overall Prevalence (95% CI)	Prevalence -Multiple Infections (95% CI)
3	0.0478 (0.0470, 0.0485)	0.0169 (0.0165, 0.0174)
5	0.0083 (0.0079, 0.0086)	0.0033 (0.0031, 0.0035)
6	0.0467 (0.0459, 0.0474)	0.0155 (0.0150, 0.0159)
7	0.0221 (0.0216, 0.0227)	0.0074 (0.0071, 0.0077)
9	0.0956 (0.0946, 0.0966)	0.0260 (0.0254, 0.0266)
10	0.0151 (0.0146, 0.0155)	0.0053 (0.0050, 0.0056)
11	0.0019 (0.0018, 0.0021)	0.0007 (0.0006, 0.0008)