

Evaluation of Human Papillomavirus Type Replacement Postvaccination Must Account for Diagnostic Artifacts: Masking of HPV52 by HPV16 in Anogenital Specimens

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Abstract

It has been hypothesized that, following a reduction in human papillomavirus (HPV) vaccine-targeted genotypes, an increase in prevalence of other HPV types may occur due to reduced competition during natural infection. Any apparent postvaccination increase must be distinguished from diagnostic artifacts consequent to consensus PCR assays failing to detect HPV types present in low copy numbers in coinfecting specimens (under the assumption that with a drop in vaccine-preventable types there may be increased detection of previously "masked" types). We reanalyzed anogenital specimens to evaluate unmasking of HPV52 that may be caused by elimination of HPV16. Using highly sensitive type-specific real-time HPV52 PCR, we retested 1,200 anogenital specimens (all HPV52 negative according to consensus PCR assays) from six epidemiologic studies (200 specimens/study; 100 HPV16⁺/study). Multivariate logistic regression, with adjustment

for age and number of sexual partners, was used to evaluate the association between HPV16 positivity and detection of HPV52. In our pooled analysis ($n = 1,196$), the presence of HPV16 was positively associated with HPV52 detection [adjusted OR, 1.47; 95% confidence interval (CI), 0.76–2.82]. In our separate (study specific) analyses, a statistically significant association was observed in one study that included HIV-infected males (HIPVIRG study; adjusted OR, 3.82; 95% CI, 1.19–12.26). We observed a positive association between HPV16 viral load (tertiles) and detection of HPV52 (P for trend = 0.003). These results indicate that diagnostic artifacts, resulting from unmasking of HPV52, may occur in some settings in the evaluation of HPV type replacement. Additional studies exploring the extent and severity of unmasking are needed. *Cancer Epidemiol Biomarkers Prev*; 24(1); 286–90. ©2014 AACR.

Introduction

Infection with oncogenic human papillomavirus (HPV) types is necessary for cervical cancer development. Currently, two commercially available vaccines offer protection against the two major oncogenic HPV types (16 and 18) and associated lesions, but only one of these vaccines also protects against HPV types 6

and 11, which are responsible for the majority of anogenital warts (1).

Vaccination has begun to reduce the prevalence and burden of vaccine-targeted HPV types (2, 3); however, as this occurs, there is concern that abrogation of selective pressure could lead to an increase in the prevalence of other nonvaccine HPV types. This phenomenon, referred to as "type replacement," may occur as a result of one or more HPV types becoming unrestricted in their ability to occupy the niche originally taken by vaccine-targeted types during natural infection. However, an apparent rise in nonvaccine HPV types may occur due to diagnostic artifacts if there is competition between vaccine and nonvaccine HPV types for reagents (e.g., primers) in consensus-primer polymerase chain reaction (PCR) assays. In this situation, it is possible that prevalent nonvaccine types may be undetected. For instance, if a coinfecting specimen contains a much higher number of HPV16 genome copies, then it may overwhelm the minority type(s) during PCR amplification, and as a result, the specimen may be erroneously labelled as negative for the minority type(s). Therefore, a reduction in the rate of detection of vaccine types postvaccination in genital specimens may lead to an apparent increase in some HPV types that were previously masked. Such unmasking effect could be mistaken for type replacement. HPV16 is currently the most common HPV type globally and is often present in high viral load concentrations. Thus, compared with other genotypes targeted by vaccination (HPVs 6, 11, and 18), reductions in HPV16

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prevalence postvaccination will likely be most responsible for unmasking. Previous International Agency for Research on Cancer (IARC) studies evaluating HPV type interactions, among specimens from both men and women, suggested that diagnostic artifacts may explain the apparent clustering of certain HPV infections, e.g., HPV52 with other types (4, 5).

Recently, unmasking has been cited as a possible explanation for negative vaccine efficacy observed in one trial for some endpoints involving specific HPV genotypes, particularly HPV52 (6). In addition, two studies evaluating the population effect of vaccination in the United States and Scotland recently revealed slight increases in certain HPV types, including HPV52 (2, 3). PCR does not always amplify different DNA segments with equal efficiency and reduced sensitivity of consensus primer PCR (compared with type-specific or multiple primer systems) for detection of certain HPV types in coinfecting specimens has been reported and found to be associated with lower viral DNA load (7–10). Recently, one study found that in specimens coinfecting with HPV16 and either HPV18, -51, -52, or -58, consensus PCR often failed to detect the latter types, particularly at lower viral loads and for HPVs 51 and 52 (9). Therefore, despite lack of evidence of HPV type competition from most epidemiological studies (11), results from these studies comparing different PCR assays (7–10), as well as the recent report of negative vaccine efficacy against HPV52-associated cervical neoplasia (6) is what motivated us to focus our evaluation on unmasking of HPV52. It is important to explore whether increases in the prevalence of HPV52 and other genotypes observed following vaccination may be the result of true type replacement, or an artifact of unmasking.

Our objective was to explore the potential for unmasking of HPV52 attributable to a reduction in HPV16 postvaccination. We investigated whether detection of HPV52 using a sensitive type-specific PCR assay varies according to HPV16 positivity and viral load among specimens originally HPV52 negative.

Materials and Methods

Study design and specimen selection

Specimens were available from the following studies: Ludwig-McGill cohort study (12), HPV Infection and Transmission among Couples through Heterosexual Activity (HITCH) study (13), McGill-Concordia cohort study (14), Biomarkers of Cervical Cancer Risk (BCCR) case-control study (15), Canadian Cervical Cancer Screening Trial (CCCaST; ref. 16), and the Human Immunodeficiency and Papilloma Virus Research Group (HIPVIRG) study (17). Each of these studies was approved by their respective institutional review boards. Informed consent was obtained from all participants before enrolment.

In total, 1,200 anogenital specimens from 1,000 women and 200 men were selected for retesting using HPV52 type-specific PCR on the basis of previous testing done using consensus-primer PCR. From each of the aforementioned six studies (12–17), an equal number of specimens ($n = 200$; all HPV52 negative) were randomly selected on the basis of the following criteria. Half of the specimens ($n = 100$) were positive for HPV16, and the other half were negative for HPV16. Because all anogenital HPV types share a common transmission route, subjects with HPV16 (or any other HPV type) would also be at higher risk of HPV52 infection. Thus, to avoid major confounding, we selected for retesting only HPV-positive specimens. Among HPV16-negative specimens, half ($n = 50$) were positive for an HPV type phylogenetically related to

HPV16 (α -9 species; except HPVs 16 or 52) and the other half were positive for some other non α -9 HPV type. This strategy ensured that we could later explore if there was a difference in HPV52 detection between these two HPV16-negative groups.

Laboratory assessments

Self or provider-collected anal, cervical, or cervicovaginal specimens were obtained using swabs, cytobrush, or spatula, according to the parent study's protocol. HPV DNA testing and genotyping was performed in the original studies with consensus primer assays (L1 PGMY or MY09/11 and hybridization with oligonucleotide probes and restriction fragment length polymorphism analysis, linear array, or line blot assay), which detect 27 to 40 different HPV types. For the present study, specimens were retested (blinded to HPV16 status) using a type-specific, real-time HPV52 PCR, which is capable of detecting as few as 10 HPV52 copies per assay (18). HPV16 viral load was quantified according to a well-established real-time PCR protocol (19) and expressed as the number of HPV DNA copies per cell.

Statistical analyses

Logistic regression was used to estimate odds ratios (OR) and associated 95% confidence intervals (CI) for the effect of HPV16 positivity on HPV52 detection. Separate analyses were performed for each study adjusted for age and lifetime number of sexual partners (multivariate model; covariates based on *a priori* knowledge), as well as pooled across studies (with adjustment for study in both crude and adjusted models). The CCCaST trial included participants from St. John's (Newfoundland) and Montreal (Quebec). Unfortunately, women from the St. John's site did not provide information on sexual history, which led to the exclusion of some specimens in our fully adjusted models ($n = 76$). By eliminating adjustment for sexual history as part of our sensitivity analyses, we were then able to include all CCCaST specimens in our pooled analysis. Analyses restricted to cervical/cervicovaginal specimens from female subjects (i.e., excluding anal specimens from male HIPVIRG participants) were also performed.

Logistic regression was also used to evaluate the effect of HPV16 viral load on HPV52 detection. For each study, HPV16 viral load was categorized into study-specific tertiles (low, medium, high). We estimated ORs for each tertile with the HPV16-negative group as the reference category. Similar sensitivity analyses as above were performed in our evaluation of the effect of HPV16 viral load on unmasking of HPV52.

Results

Among the 1,200 specimens selected for HPV52 retesting, 1,196 had sufficient beta-globin and were evaluable. In total, 49 specimens tested positive for HPV52 and the majority (30 of 49) were detected among the HPV16-positive group (Table 1). Focusing on HPV16-negative specimens, detection of HPV52 was similar between the group containing α -9 HPV types and the group that contained other (non α -9) HPV types (11 of 300 versus 8 of 298, respectively).

Across all studies, the average number of HPV types detected among HPV16-positive and HPV16-negative specimens was 2.8 and 2.4, respectively. Accounting for age and lifetime number of sexual partners, additional HPV types present within specimens was associated with an 18% increase in HPV52 detection. Overall, we observed a pooled adjusted OR of 1.47 (95% CI, 0.76–2.82)

Table 1. Association between HPV16 status and HPV52 detection based on retesting of selected cervical/anal specimens using HPV52 type-specific PCR^a

Study	Years (recruitment and follow-up)/ study population	HPV52 ⁺ specimens/total specimens, N			OR (95% CI) HPV16 ⁺ vs. HPV16 ⁻ (reference)	
		HPV16 ⁺	HPV16 ⁻ (HPV ⁺ , α-9 type)	HPV16 ⁻ (HPV ⁺ , not α-9 type)	Crude	Adjusted ^b
Ludwig-McGill	1993-05; low-income females, 18-60 y, São Paulo, Brazil	0/98	2/50	1/49	N/E	N/E
McGill-Concordia	1996-02; female students, 17-45 y, Montreal, Canada	2/100	2/50	0/50	0.99 (0.14-7.17)	0.97 (0.13-7.11)
HITCH ^c	2005-13; female students with a male partner, 18-25 y, Montreal, Canada	3/100	0/50	0/50	N/E	N/E
BCCR	2001-09; females with/without precancerous cervical lesions, 18-75 y, Montreal, Canada	6/100	2/50	1/49	2.04 (0.50-8.40)	2.14 (0.47-9.57)
CCCaST	2002-06; females screened for cervical cancer, 30-69 y, Montreal/St. John's, Canada	4/100	2/50	5/50	0.55 (0.16-1.95)	0.43 (0.11-1.71)
HIPVIRG ^d	2002-08; MSMs with HIV, 21-67 y, Montreal, Canada	15/100	3/50	1/50	4.24 (1.35-13.25)	3.82 (1.19-12.26)
All studies ^e		30/598	11/300	8/298	1.62 (0.90-2.92)	1.47 (0.76-2.82)
All studies ^e (HIPVIRG excluded)		15/498	8/250	7/248	1.00 (0.48-2.07)	0.82 (0.35-1.93)

NOTE: Test for heterogeneity between studies: $P = 0.08$.

Abbreviations: MSM, men who have sex with men; N/E, not able to estimate.

^aAll specimens were originally HPV52 negative in the source studies according to consensus primer PCR HPV DNA testing.

^bAdjusted for age, lifetime number of sexual partners, and study (pooled analysis), except for CCCaST study (adjusted for age only).

^cHITCH was the only study that included cervicovaginal specimens, and self- rather than provider-collected specimens.

^dHIPVIRG was the only study that included anal specimens; all others included either cervicovaginal or cervical specimens.

^eSome specimens from CCCaST study ($n = 76$) were excluded from adjusted pooled analysis because number of sexual partners information was not collected from certain subjects (St. John's study site only).

for the association between HPV16 status and HPV52 detection; however, we also observed substantial heterogeneity across studies (test for heterogeneity: $P = 0.08$). A statistically significant positive association was observed in HIPVIRG, but not in the other studies (Table 1). A negative association between HPV16 status and HPV52 detection was suggested in the CCCaST study; however, this association was not statistically significant. From the St. John's study site in CCCaST, HPV52 was detected in four of the 76 specimens, all of which were HPV16 negative. Excluding sexual history from our multivariate model, which allowed all CCCaST specimens to be included, had little impact on our results (pooled adjusted OR, 1.33; 95% CI, 0.71-2.46). However, in our pooled analysis restricted to female cervicovaginal specimens (HIPVIRG study excluded), a null association between HPV16 status and HPV52 detection was observed (Table 1).

We observed a strong positive association between HPV16 viral load (tertiles) and detection of HPV52 (Table 2, P for trend = 0.003). There was no meaningful change in our viral load results when we restricted our analysis to cervicovaginal specimens only (i.e., females without HIV infection), or when we included all

CCCaST specimens (adjustment for age only in our pooled analysis; results not shown).

Discussion

In specimens tested via consensus PCR, HPV16 positivity was associated with masking of HPV52 positivity in the HIPVIRG and BCCR studies. These two studies, unlike the others, included participants with HIV infection or high-grade cervical lesions, respectively. In general, high viral load HPV infections are more common among individuals with low immunity or cervical neoplasia, which may explain why an effect was observed in specimens from these studies, but not the others (20). Our interpretation is also supported by our results revealing a greater unmasking effect in specimens with higher HPV16 viral load.

To our knowledge, this is the first study designed specifically to evaluate the potential for an HPV type to be masked if in a specimen coinfected with HPV16. Our findings suggest that, all else being equal, elimination of HPV16 via vaccination may lead

Table 2. Association between HPV16 viral-load status and HPV52 detection based on retesting of selected cervical/anal specimens using HPV52 type-specific PCR^a

HPV16 viral load (tertiles)	HPV52 ⁺ specimens/total specimens, N							OR ^b (95% CI) All studies ^c
	Ludwig-McGill	McGill-Concordia	HITCH	BCCR	CCCaST	HIPVIRG	Total	
HPV16 ⁻	3/99	2/100	0/100	3/99	7/100	4/100	19/598	Ref
HPV16 ⁺								
Low	0/30	0/33	1/33	2/33	0/32	2/33	5/194	0.73 (0.24-2.21)
Middle	0/30	1/33	1/33	0/33	2/32	7/33	11/194	1.38 (0.55-3.45)
High	0/30	1/34	1/34	4/34	2/33	6/34	14/199	2.36 (1.08-5.14)

NOTE: Test for heterogeneity between studies: $P = 0.52$.

^aAll specimens were HPV52 negative according to consensus primer PCR HPV DNA testing.

^bAdjusted for age, lifetime number of sexual partners, and study.

^cSome specimens from CCCaST study ($n = 76$) were excluded from analysis because number of sexual partners information was not collected from certain subjects (St. John's study site only).

to some unmasking of previously undetectable infections with a type such as HPV52. Important strengths of our study were its size and the diverse study populations from which specimens were selected. Had we focused our analysis exclusively on specimens from females or disease-free individuals, we would have missed the opportunity to discover an HPV16-induced masking effect in the two aforementioned studies. A possible limitation of our study was that the HPV16-negative group remained positive for other HPV type(s). As a result, masking of HPV52 may have occurred in this group as well, causing our effect estimates to be biased toward zero. But because those with HPV16 are at much higher risk of infection with other types (including HPV52), this decision was intended to avoid confounding by sexual activity and other risk factors common to all HPV types. Despite this conservative approach, we still observed a strong and statistically significant effect in the HIPVIRG study, as well as at higher HPV16 viral loads. For our pooled analyses (Table 1; all studies and all studies excluding HIPVIRG), we also performed sensitivity analyses restricted to specimens with exactly two HPV infections (i.e., the infection on which selection was based, plus one other) but found that results were not meaningfully different (data not shown), therefore providing reassurance that confounding by sexual behavior did not bias our original results.

As investigators begin to evaluate HPV type replacement, they will rely on time point comparisons of HPV prevalence from surveys before and after vaccination. However, if an increase in HPV52 (or other HPV types) is observed postvaccination, unmasking should be suspected. On the basis of the results from this study, correction formulas for adjustment of baseline prevalence of HPV52 infection due to masking may not be necessary in all settings and will likely depend on the risk group being considered. For example, masking of HPV52 may be less common among specimens from low-risk individuals in North America. Meanwhile, in parts of sub-Saharan Africa or other high-risk regions where there is high prevalence of HIV and HPV coinfection, elimination of vaccine target types could lead to larger increases in the prevalence of HPV52 or other HPV types due to unmasking.

Globally, consensus primer PCR assays are the most common HPV DNA tests used for research and surveillance. To evaluate whether different assays perform similarly in cases of multiple HPV infection, the World Health Organization HPV laboratory network has now assembled blinded "proficiency panels," and so far results from more than 100 laboratories indicate that masking is a definite problem for some of these assays (10). In the 2010 HPV genotyping proficiency panel, samples included 16 HPV types (6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68a/68b) and across laboratories, 24 different genotyping methods were used, including Linear Array (most common), line blot assay, and type-specific real-time PCR. In both single and multiple infections, proficient typing was defined as: detection of 50 international units (genome equivalents) per 5 μ L of HPV16 and HPV18, detection of 500 genome equivalents per 5 μ L for the remaining 14 HPV types, and not more than one false-positive result (10). In two of our parent studies (Ludwig-McGill and McGill-Concordia), the MY09/11 PCR protocol was used in combination with hybridization using individual oligonucleotide probes/restriction fragment length polymorphism or reverse line blot assay, respectively. In the remaining studies, consensus primer PGM09/11 PCR was used with either linear array (HITCH and CCCaST) or reverse line blot assay (BCCR and HIPVIRG). Although linear array, which uses a cross-reactive probe to detect HPVs 33, 35, 52, and 58,

is known to have issues in its ability to accurately detect HPV52 (18, 21), this test was not used in HIPVIRG and therefore issues surrounding this cross-reacting probe cannot be responsible for unmasking that we observed in this study.

To avoid false reports of type replacement, correction formulas to account for unmasking may be useful for comparison of pre- and postvaccination HPV prevalence in certain settings. For example, focusing on HPV52, if X represents the number of newly detected HPV coinfections involving HPVs 16 and 52 using type-specific PCR, and Y represents the original number of HPV16/52 coinfections detected using consensus PCR in the population; then the basic formula to calculate type replacement to be expected resulting from elimination of HPV16, but attributable to unmasking in a specific population/risk group is $[(X)/(X + Y)] \times 100\%$, which assumes random sampling and appropriate sampling error calculations. Future studies evaluating the potential for unmasking of HPV52 and other genotypes in low- and high-risk settings will be helpful for determining the extent and severity of unmasking.

Disclosure of Potential Conflicts of Interest

M.-H. Mayrand reports receiving a commercial research grant from Merck. F. Coutlée has speakers' bureau honoraria from Merck Sharp & Dohme. E.L. Franco has received a commercial research grant from Merck and has been a consultant/advisory board member for Merck, GlaxoSmithKline, Roche, and Gen-Probe. L.L. Villa has received honoraria from speakers' bureau from Merck and has been a consultant/advisory board member for Merck. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: J.E. Tota, L.L. Villa, F. Coutlée, E.L. Franco
Development of methodology: J.E. Tota, L.L. Villa, F. Coutlée, E.L. Franco
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.E. Tota, A.V. Ramanakumar, L.L. Villa, A.N. Burchell, A. Koushik, M.-H. Mayrand, F. Coutlée, E.L. Franco
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.E. Tota, A.V. Ramanakumar, L.L. Villa, M.-H. Mayrand, F. Coutlée, E.L. Franco
Writing, review, and/or revision of the manuscript: J.E. Tota, A.V. Ramanakumar, L.L. Villa, H. Richardson, A.N. Burchell, A. Koushik, M.-H. Mayrand, F. Coutlée, E.L. Franco
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.E. Tota, H. Richardson, A.N. Burchell, E.L. Franco
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