Human Papillomavirus (HPV) Genotyping Using Paired Exfoliated Cervicovaginal Cells and Paraffin-Embedded Tissues To Highlight Difficulties in Attributing HPV Types to Specific Lesions

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Defining type-specific human papillomavirus (HPV) infections within cervical tissues is important for understanding the pathogenesis of cervical neoplasia and assessing the effectiveness of prophylactic vaccines with limited type-specific spectra. We compared HPV DNA-testing results from 146 matched exfoliated-cell- and formalin-fixed-tissue specimens collected by cervicovaginal lavage (CVL) within 90 days of each other from women with histologically confirmed cervical intraepithelial lesions (CIN). The CVL specimens were HPV typed using a MY09/11 L1 consensus primer PCR method followed by dot blot hybridization. The tissue specimens were HPV typed using an SPF10 line probe assay HPV detection system. Of the 146 specimen pairs with evidence of CIN in the tissue, 91.8% were positive for one or more HPV types in both the tissue and cellular specimens. Tissue sections were more likely to be HPV negative (P < 0.01). Typing directly from tissue sections resolved multiple infections detected in exfoliated cells to a single HPV type in only 46.9% of cases. Combined use of both specimen types to attribute lesions to HPV type 16 (HPV-16) and/or -18 led to 43.1% attributed to HPV-16 and/or -18 by both specimen types and 19.9% attributed to HPV-16 and/or -18 by one, but not both, specimen types. Unambiguous attribution of cervical lesions to a single, specific HPV type remains a difficult proposition. Use of multiple specimen types or the development of highly sensitive and robust in situ hybridization HPV-testing methods to evaluate the certainty of attribution of lesions to HPV types might provide insights in future efforts, including HPV vaccine trials.

Infection with one of approximately 15 oncogenic types of human papillomavirus (HPV) has been established as a necessary but insufficient cause of cervical cancer (12). Although HPV typing performed by testing DNA extracted from exfoliated cervicovaginal cells is an accurate technique for demonstrating HPV infections, this approach does not meet growing needs to topographically define the specific areas of the cervix that harbor the virus. Among HPV-positive women, 20 to 40% are infected with multiple HPV types. Some HPV types detected by testing exfoliative specimens may reflect vaginal rather than cervical infections, thus underscoring the need for methods that permit localization of viral infection within specific compartments of the cervical mucosa.

Clinical trials conducted to test the efficacy of prophylactic vaccines that target two oncogenic HPV types, HPV-16 and HPV-18 (one of the vaccines also targets two nononcogenic HPV types, HPV-6 and HPV-11), have created an immediate need for HPV typing of tissues to colocalize HPV infections and their associated lesions. In most vaccine trials, prevention of cancer precursors (i.e., cervical intraepithelial neoplasia [CIN] grade 2/3) related to HPV-16 and/or -18 is an important end point. Given that prophylactic vaccines are expected to provide minimal protection against untargeted types, it is desirable in trial to accurately HPV type all CIN 2/3 lesions (7). This is particularly true in the context of multiple HPV infections, where testing of exfoliated cervical cells cannot define the specific HPV type contained in the histologic lesions of interest.

We report here a comparison of tissue-based HPV testing using the SPF10 line probe assay (LiPA) detection system against previously available exfoliated-cell-based HPV-testing results using the well-characterized MY09/11-HMB01 amplification with oligonucleotide dot blot hybridization (11). The SPF10 LiPA is a broad-spectrum short-fragment PCR (SPF10 PCR) that recognizes most genital-tract HPV types by amplifying a conserved 65-bp fragment of the L1 region of the HPV genome, the smallest amplicon of any HPV DNA-typing system available (9, 10). Accordingly, it was expected that this system would provide the highest probability for HPV amplification from DNA extracted from fixed tissues. The MY09/11 and SPF10 consensus primer systems target a common region of the L1 open reading frame and have comparable sensitivities, with only slight type-specific differences in amplification efficiency (3, 6, 17; reviewed in reference 5). The specific aims...
of our study were to (i) determine the overall concordance between results obtained from these two specimen types/test-
ing methods and (ii) estimate the frequency with which HPV typing of tissue specimens helps clarify the type-specific eti-
ology of the lesions involved.

MATERIALS AND METHODS

Study population. This study was conducted within the 20,000-woman Port-
land Kaiser Permanente Cohort Study of HPV infection and cervical neoplasia.
The Portland cohort study has been previously described (11, 14). Briefly, we en-
rolled women from 1989 to 1990 from one of seven routine Pap smear screen-
ing clinics at Portland Kaiser-Permanente and followed them prospectively by
routine cytology for up to 10 years. Informed consent was obtained at institu-
tional review boards at Kaiser Permanente and at the National Institutes of
Health. At enrollment and during follow-up, HPV testing of exfoliated-cell
specimens (cervicovaginal lavage [CVL] specimens) was conducted with a PCR-
based method using MY09/11 primers and dot blot typing (11).

We obtained 486 formalin-fixed and paraffin-embedded tissue specimens from
a total of 460 histologically confirmed CIN cases identified during the enrollment
or follow-up phase of our cohort study. This cohort is based on a population
of women expected to be HPV DNA positive. For the present study, we selected
211 of the 486 women for whom tissue blocks were available and who had
exfoliated-cell specimens that were collected within 90 days prior to the tissue
collection date. Three specimens utilized during the optimization phase of
this study were not available for final testing as part of this effort. The present
study was restricted to the remaining set of 208 women. Hematoxylin and
cosin (H&E) review was performed to confirm the presence of lesions in the
final flanking section following those collected for PCR. We were able to review
slides for 195 of the 208 women (93.8%) included in our study; slides for the
remaining 13 women could not be located and were excluded from the analysis.
We confirmed the presence of lesional material in 74.9% (n = 146 out of 195) of
the specimens evaluated. The 49 specimens for which lesional material was
absent in the H&E slides flaking the tissue ribbons used for HPV genotyping
were excluded from further evaluation. The median time between collection of
the exfoliated-cell and tissue specimens for the 146 specimens pairs included
in this evaluation was 25 days (56% were collected within 1 month; range, 0 to 87
days).

Tissue sectioning. Sandwich-based sectioning of blocks was performed as
follows. After resurfacing of the block, one 4- to 5-μm section was cut for
histological evaluation. Three 10-μm ribbons were then prepared, and each
section was stored in separate storage vials at controlled room temperature.
This was followed by three 4- to 5-μm sections mounted on slides (unstained). A final
4- to 5-μm section was then cut and mounted on a slide and subsequently H&E
stained for evaluation. PCR-safe precautions were taken to minimize the risk of
contamination during tissue processing. They included changing of gloves be-
tween specimens, careful cleaning of the microtome and blade replacement
between specimens, and use of disposable toothpicks to transfer tissue
ribbons to their storage vials. Sectioning was performed at a facility that does not
perform HPV testing.

Histologic evaluation. H&E slides immediately adjacent to the tissue ribbons
used for HPV DNA typing were reviewed by the study pathologist (M. Sherman)
for the presence or absence of lesions to ensure that the tissue selected for viral
typing contained lesional material. Further classification was made with respect
to severity (CIN 1 versus CIN 2+) and size, qualitatively assessed as small or
large. Tissue microdissection was not performed, given that our objective was to
evaluate whether virological testing performed on the tissue block containing the
lesion of concern would improve identification of the causal agent compared to
large. Tissue microdissection was not performed, given that our objective was to
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Analytic methods. For each woman, we evaluated overall HPV positive/neg-
ative percent agreement between exfoliated-cell- and tissue-based HPV test
results. Among women with global HPV concordance, we evaluated HPV type-
specific agreement by calculating both complete agreement (i.e., agreement on
all detected HPV types) and partial agreement (i.e., agreement on at least one
but not all HPV types detected). We additionally reported individual HPV
type-specific agreement, using as the denominator the total number of HPV
infections detected (i.e., at the level of analysis of infections rather than women).
All type-specific analyses were restricted to genotypes detected by both systems
in order to more directly infer comparability by specimen type (the HPV types
detected by both systems were 6/11, 16, 18, 31, 33, 35, 39, 40, 45, 51, 52, 53, 56,
58, 66, and 68). McNemar's chi-square was calculated to test for significant
trends in the pattern of disagreement. We stratified by whether single or multiple
infections were observed for either specimen type. Analyses stratified by lesion
size (small versus large), grade of disease (CIN 1 versus CIN 2+), and time
between exfoliated-cell and tissue collection (<14 days, <30 days, and >30 days)
did not materially affect the conclusions and are not reported here.

When we evaluated the impacts of different approaches that might be em-
ployed to define HPV-16- and/or -18-associated lesions, the following strategies
were considered: (i) HPV-16 and/or -18 attribution defined solely on the basis of
results obtained from exfoliated-cell specimens; (ii) HPV-16 and/or -18 attribu-
tion defined solely on the basis of results obtained from tissue specimens; (iii)
HPV-16 and/or -18 attribution defined primarily using tissue results, with exfo-
liated-cell testing results used to clarify attribution for negative tissue specimens;
(iv) HPV-16 and/or -18 attribution defined primarily using exfoliated-cell results,
with tissue results used to clarify instances where multiple HPV types were
detectable using the exfoliated-cell specimen; and (v) HPV-16 and/or -18 attribu-
tion defined based on results from both specimens, where a definitive HPV-16
and/or -18 attribution was made when both specimens contained HPV-16 and/or
-18 and a possible HPV-16 and/or -18 attribution was made when one specimen
type, but not the other, contained HPV-16 and/or -18.

RESULTS

Histological evaluation of tissue specimens. Among the 146 specimens with discernible lesions, CIN 1 was observed for

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Overall agreement between exfoliated-cell- and tissue-based HPV testing. The overall agreement of HPV status (positive/negative) was 91.8% between exfoliated-cell and tissue pairs (Table 1). All subjects tested positive in at least one of the two specimens, consistent with the study population consisting of women with rigorously reviewed CIN and the use of PCR assays targeting a wide range of HPV types. Of the 12 HPV-discordant samples, 11 (7.5%) exfoliated-cell specimens were HPV positive but negative on the paired tissue specimen and one tissue specimen (0.7%) tested HPV positive with a negative result for the paired cellular specimen. (McNemar’s P value = 0.004). Subject ages and the human DNA equivalents based on ERV-3 quantitation were similar in HPV-negative tissue specimens (mean = 221 cell equivalents; range, 12 to 818) and HPV-positive tissue specimens (mean = 141 cell equivalents; range, 0.25 to 1,250).

HPV type-specific agreement between exfoliated-cell- and tissue-based HPV testing. In analyses restricted to positive specimens, complete HPV type agreement was observed for 44.0% of pairs, and 37.3% exhibited partial HPV type agreement (data not shown). Of the 281 total HPV types detected (i.e., counting each HPV type detected rather than each woman as the analytical unit), 48.8% agreement was observed. The pattern of discordant results (tissue positive/exfoliated cell negative versus tissue negative/exfoliated cell positive) favored HPV detection in the exfoliated-cell sample for most types (Table 2), though the differences were rarely statistically significant (significance was defined as a McNemar’s P value of <0.05). An opposite trend was observed for HPV-52, which was detected in tissues significantly more often than in the paired exfoliated cells (P = 0.001).

Table of data:

<table>
<thead>
<tr>
<th>Tissue specimen condition</th>
<th>No. of paired exfoliated-cell specimens</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPV positive</td>
<td>HPV negative</td>
</tr>
<tr>
<td>HPV positive</td>
<td>134</td>
<td>1</td>
</tr>
<tr>
<td>HPV negative</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>145</td>
<td>1</td>
</tr>
</tbody>
</table>

* Overall agreement: 91.8%; McNemar’s chi-square (P value): 8.3 (0.004).
-18 ranged from 43.1% to 63.0%, depending on the strategy employed. For the strategy that considered both specimen types equally when defining attribution (strategy 5 in Table 4), we observed that 43.1% of lesions were definitively attributable to HPV-16 and/or -18 infection and 37.0% of lesions were definitively not attributable to HPV-16 and/or -18 infection, whereas the remaining 19.9% of lesions were inconclusively attributable to HPV-16 and/or -18 infection.
DISCUSSION

We found good overall (91.8%) and type-specific (43.3% complete; 81.4% complete or partial) concordance between 146 paired specimens for which we could confirm evidence of CIN in the tissue used for typing. When the agreement was restricted to vaccine-targeted HPV-16 and/or -18 infections, we found similarly high agreement (80.1%). In this analysis, HPV-16 and/or -18 discordance was not associated with sample type (P = 0.85). These observations suggest that, in the evaluation of vaccine efficacy based on HPV-16 and/or -18 detection, tissue and exfoliated-cell specimens yield similar results (strategy 1 versus strategy 2 [Table 4]) and that the combination (strategy 5 [Table 4]) yields maximal detection of both putative and definitive infection with vaccine-related types. Our results highlight the fact that neither tissue-based or exfoliated-cell-based HPV genotyping results in absolute clarification of HPV-16- and/or -18-associated lesions.

Theoretically, directed sampling of the cervix by targeted biopsy would allow detection of a subset (ideally one) of the many potential foci of HPV infection in the cervicovaginal tract. In this analysis, while we did see overall fewer multiple infections and genotypes in tissue specimens than in exfoliated-cell specimens, we were unable to resolve lesions to a single HPV genotype in the tissue specimen in over one-half of the cases where the exfoliated-cell specimen showed multiple HPV types. These results suggest little gain in clarity when employing tissue-based HPV detection, while considerable complexity and expense are added.

Despite the good overall level of agreement observed, the following complexities in using tissue and/or exfoliated-cell specimens for HPV DNA testing were encountered in our study. First, approximately 25% of the paired specimens in our study were excluded from our analysis because we did not observe any residual CIN in the tissue available for testing. This was not unexpected, given that CIN lesions are often small and that priority must be given to histologic evaluation over HPV typing.

Second, we observed a lower overall HPV prevalence (any type versus none) in tissue sections (92.5%) than in exfoliated-cell specimens (99.3%; McNemar’s P = 0.004). The mean quantity of amplifiable human DNA in HPV-negative tissue specimens was slightly higher than that observed in the HPV-positive tissue specimens, suggesting that this discordance was not a reflection of DNA recovery or degradation or PCR inhibition. To the extent that the HPV was present in low copy number in the tissue digest, the difference could also be explained by stochastic sampling error.

Third, HPV types detectable in tissue were not always evident in the matched exfoliated-cell specimen, as would have been expected, since biopsy specimens should represent a focused sample that represents a subset of cells sampled from CVL. This finding indicates that even in instances where a single HPV type is detected in exfoliated cells, one cannot be assured that the type detected matches the HPV type found in the lesion. Some of these typing discrepancies could potentially be explained by differences in the two testing systems used in this analysis (3, 6, 17; reviewed in reference 5). The retrospective nature of this study precluded retesting of the lavage samples using the SPF10 primer system, and the degradation of DNA from the fixed tissue sections precluded testing with the large-fragment PCR generated by MY09/11. However, the sensitivity limitations reported in comparing MY09/11 and PGMY09/11 primers or TaqGold polymerase and regular Taq polymerase were largely restricted to samples collected from women without cytologic abnormalities and therefore with low viral loads (3, 6). Because this analysis was restricted to women with biopsies resulting from suspicious Pap smear results, we do not believe that the sensitivity differences previously reported as a result of using MY09/11 with regular Taq polymerase resulted in a significant amount of discordance. In fact, the type-specific agreement was good between the tissue-SPF and lavage-MY09/11 paired samples, with most types favoring detection in the CVL, an effect opposite the expectation under an assumption of limited sensitivity of the MY09/11-plus-Taq assay. The exception was a significant increase in the detection of HPV-52 (a type shown to be poorly amplified with the MY09/11 primers [6]) in tissue sections tested by SPF10 versus exfoliated cells tested with MY09/11, suggesting that a proportion of these discordant pairs (n = 12) were HPV-52 false-negative results from the MY09/11-tested exfoliated-cell specimens. We also note that the agreement observed in this study is remarkably similar to that in an earlier study that used SPF10 primers for HPV detection from both the exfoliated-cell and tissue specimens, further supporting our argument that the use of two primer systems does not introduce an important bias in the main conclusions of our study (13).

(i.e., one specimen type indicated HPV-16/18 attribution while the other did not).

<table>
<thead>
<tr>
<th>Tissue specimen condition</th>
<th>No. of paired exfoliated-cell specimens</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPV-16/18 positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HPV-16/18 negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV-16/18 positive</td>
<td>63</td>
<td>14</td>
<td>77</td>
</tr>
<tr>
<td>HPV-16/18 negative</td>
<td>15</td>
<td>54</td>
<td>69</td>
</tr>
<tr>
<td>Total</td>
<td>78</td>
<td>68</td>
<td>146</td>
</tr>
</tbody>
</table>

* Overall agreement: 80.1%; McNemar’s chi-square (P value): 0.03 (0.85).

Table 3. Overall HPV-16 and/or -18 concordance between paired exfoliated-cell and paraffin-embedded tissue specimens

Table 4. Comparison of alternative definitions of HPV-16/18 attribution of lesions

<table>
<thead>
<tr>
<th>Attribution based on strategy</th>
<th>% Negative</th>
<th>% Positive for non-HPV-16/18</th>
<th>% Positive for HPV-16/18</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.7</td>
<td>45.9</td>
<td>53.4</td>
</tr>
<tr>
<td>2</td>
<td>7.5</td>
<td>39.7</td>
<td>52.8</td>
</tr>
<tr>
<td>3</td>
<td>0.0</td>
<td>44.5</td>
<td>55.5</td>
</tr>
<tr>
<td>4</td>
<td>0.0</td>
<td>54.1</td>
<td>45.9</td>
</tr>
<tr>
<td>5</td>
<td>0.0</td>
<td>37.0</td>
<td>19.9 (possible); 43.1 (definitive)</td>
</tr>
</tbody>
</table>

* Strategies: 1, exfoliated-cell specimen only; 2, tissue specimen only; 3, tissue specimen primary (exfoliated-cell specimens to clarify HPV-negative tissues); 4, exfoliated-cell specimens primary (tissue specimens used to clarify exfoliated-cell specimens with multiple HPV types); 5, exfoliated-cell and tissue specimens both primary.

* Classified as possible if HPV-16/18 were detected in only one specimen in a pair and as definitive if HPV-16/18 were detected in both specimens in a pair.
Another potential limitation of our study is the use of exfoliated-cell and tissue specimens that were not collected concurrently. It is known that HPV infections are transient (15) and that CIN has a high potential to regress over time (1, 16). To minimize discrepancies between specimen types resulting from changes in HPV status over time, we restricted our study to specimens pairs collected within 90 days of each other. Furthermore, we conducted analyses restricted to specimens collected within 30 and 14 days of each other, and the conclusions remained similar. This suggests that while changes in HPV status over time might account for some of our findings, they are unlikely to completely explain our results.

Finally, the exfoliated-cell specimens used in our study were collected via lavage. This differs from the more commonly used swab and/or scrape collection methods for HPV DNA testing. Formal comparisons between CVL- and scrape-based exfoliated-cell collection methods have indicated that these methods are largely comparable for PCR-based HPV DNA testing, suggesting that the use of lavage specimens as our source of exfoliated cells is unlikely to explain our results (4).

In summary, while our results should be interpreted as a lower bound of agreement between exfoliated-cell- and tissue-based sampling methods, the inability to resolve infections to a single type using tissue versus exfoliated cells is unlikely to have been significantly affected by our study limitations.

Ultimately, the preference for tissue versus exfoliated-cell specimens as a means to clarify HPV types associated with specific lesions in vaccine trials and natural-history studies will depend on the scientific question to be answered. Since HPV vaccination is believed to prevent infection, not disease when infection is present, persistent detection of an HPV type contained in the vaccine in an exfoliated-cell specimen even in the absence of tissue-detected HPV of the same type might inherently represent a vaccine failure on a primary level in the absence of contamination from recent sexual activity. Thus, use of viral outcomes (particularly long-term HPV persistence, which is known to correlate with risk of disease progression) in these trials might be desirable and would eliminate the need to colocalize HPV infections to specific cells within the cervix. In addition, use of highly specific and sensitive type-specific TaqMan quantitative PCR tests targeting vaccine-associated HPV types could be readily employed. However, as long as vaccine type-associated lesions are the primary end points for vaccine efficacy, the use of limited-spectrum type-specific assays will not add clarity in the context of multiple infections, since unmeasured coinfections might still have resulted in the diagnostic lesion. For natural-history studies, it is important to consider that HPV types detectable using exfoliated-cell specimens that are not observed by tissue specimen testing does not rule out HPV-associated lesions missed by colposcopically directed biopsy (8). For those studies where colocalization of HPV infection to specific lesions is required to achieve specific scientific objectives, the development of type-specific, highly sensitive, and robust in situ hybridization HPV-testing methods might be desirable.

REFERENCES