Abstract

In large active cohort studies of women investigating human papillomavirus (HPV) and cervical neoplasia, many women will be HPV-negative at all time points and testing of all their cervical specimens is an inefficient use of laboratory resources. The aim of this pilot study was to evaluate whether pooling cervical specimens from the same woman might provide a useful pretest of specimens from women unlikely to have high-grade cervical neoplasia or significant HPV exposure. We selected women (n = 187) participating in the Guanacaste Project for whom we already had HPV testing data on all their specimens from multiple visits (median = 8 visits), who were HPV DNA-negative at enrollment and at their 5- to 7-year exit from the cohort, and had no evidence of high-grade cervical neoplasia. Equal aliquots of cervical specimens from these women were pooled to create a proportional pooled specimen. Aliquots of pooled specimens were tested in a masked fashion by MY09/11 L1 consensus primer PCR. Second aliquots of some pooled specimens were included to assess the reliability of pooled testing. Results were compared with the predicted (expected) results based on the obtained test results of the individual specimens collected at interim visits. There was good overall agreement between observed and expected HPV DNA positivity, with a κ of 0.63 [95% confidence interval (95% CI), 0.51-0.75] and a percent agreement of 83.4% (95% CI, 77.3-88.5%) although the HPV DNA positivity in the pooled specimen was less than expected (P = 0.001). The agreement between observed and expected HPV DNA positivity was related to the number of aliquots pooled, suggesting that positivity was related to viral genome concentrations. The κ and percent agreement for intra-batch reliability of testing pooled specimens were 0.68 (95% CI, 0.53-0.84) and 84.3% (95% CI, 74.7-91.4%), respectively. We conclude that pooling specimens and testing by PCR may be useful for discriminating HPV DNA-positive from completely negative specimen sets in women who are likely to have been HPV DNA-negative.

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Introduction

Human papillomavirus (HPV) infection has been established as the cause of virtually all cervical cancer (1-3). Large cohort studies (4-6) are now in place to investigate patterns of HPV infection to understand the phenomena of viral persistence and progression from infection to cervical pre-cancer and cancer versus viral clearance. These studies incorporate active follow-up with multiple visits and the collection of cervical specimens for HPV DNA testing.

Most sexually active women are exposed to HPV in their lifetime (7). However, even in studies of populations at high risk of cervical pre-cancer and cancer, many women will not be HPV DNA-positive during the fraction of their lifetime under observation in the cohort. Testing of their cervical specimens is likely to result in mainly negative test results.

In the Guanacaste Project (4, 8), a population-based cohort study of HPV natural history and the development of cervical neoplasia, we have recently finished the 7-year follow-up phase of the study. Having completed HPV testing by MY09/11 L1 consensus primer PCR of specimens collected at enrollment and exit, we have initiated longitudinal testing of the specimens collected at interim visits to correlate patterns of HPV with multiple outcomes. However, as described, many women were negative for cervical HPV DNA at their first and last visit and therefore were unlikely to have significant disease or even prolonged viral persistence. Yet we are interested in evaluating transient patterns of HPV infection. To potentially ease the burden of laboratory testing, we evaluated whether pooling cervical specimens from the same woman for HPV DNA testing was feasible. If the loss in sensitivity of testing pooled specimens is small, and if HPV prevalence is low, only specimens of subjects in whom the pooled specimens tested positive would be tested individually. Such an approach might provide a convenient method by which we could screen for the subset of women for complete HPV determination and significantly reduce the number of tests done by the laboratory. We thus tested whether pooling cervical specimens from the same woman and testing their pooled specimen by PCR might be a useful screen for a history of HPV DNA positivity.

Materials and Methods

Study Population. The Guanacaste Project is a National Cancer Institute–sponsored, National Cancer Institute–, and...
overnight with radiolabeled generic probes for HPV (HPV 11, specimens tested. DNA-positive control, and a 100-cell copy of HuH7 HPV copy SiHa HPV DNA-positive control, a 2-cell copy SiHa HPV cycles with a final extension at 72°C for 5 minutes. A 100-cell seconds, and extension at 72°C for 60 seconds for a total 40

STM specimen was lysed, and the specimen DNA was previously described (10, 11). Briefly, a 100-

Data and Specimen Collection. Enrollment and follow-up visits were very similar and consisted of an interview and a pelvic examination (4, 8). During pelvic exams, two collections of exfoliative cervical cells were done, one for the conventional and liquid-based cytology and a second that was placed into 1.0 mL of specimen transport medium (STM; Digene) and stored frozen until used for HPV DNA testing.

HPV Testing. HPV DNA was detected using MY09/11 L1 consensus primer PCR with AmpliTag Gold polymerase as previously described (10, 11). Briefly, a 100-μL aliquot of the STM specimen was lysed, and the specimen DNA was precipitated by ammonium acetate/ethanol solution and then pelleted by centrifugation. The DNA pellet was suspended in 10 mmol/L Tris (pH 8.0) with 0.1 mmol/L EDTA and stored frozen until use. Thermocycling conditions included initial denaturation at 95°C for 9 minutes; thereafter, each cycle consisted of 95°C for 60 seconds, 55°C annealing for 60 seconds, and extension at 72°C for 60 seconds for a total 40 cycles with a final extension at 72°C for 5 minutes. A 100-cell copy SiHa HPV DNA-positive control, a 2-cell copy SiHa HPV DNA-positive control, and a 100-cell copy of HuH7 HPV DNA-negative control were included with every batch of 45 specimens tested.

PCR products were analyzed by gel electrophoresis and then transferred to nylon filters. The filters were hybridized overnight with radiolabeled generic probes for HPV (HPV 11, 16, 18, 51, 52, and 81 combined). Thereafter, HPV PCR products were typed using dot blot hybridization with type-specific oligonucleotide probes for HPV types 2, 6, 11, 13, 16, 18, 26, 31 to 35, 39, 40, 42 to 45, 51 to 59, 61, 62, 64, 66 to 74, 81 to 85, 82v (AE2), 89, AE9, and AE10 (10, 11). Probes for HPV Types 2, 13, 34, 42 to 4, 57, 62, 64, 69, 74, 82, and AE9 were combined in dot blot hybridizations for detection of less common types (dbmix). DNA-positive specimens were tested twice to evaluate HPV DNA testing results, based on the assumption that a single positive individual specimen included in a pool would result in the positive test for the pooled specimen (expected positive). The same statistics were used to evaluate the intra-batch reliability. Finally, 100-μL aliquots from 21 randomly selected HPV DNA-positive individual specimens and 45 randomly selected HPV DNA-negative individual specimens were used as quality control specimens to assess reproducibility of the assay. The 336 aliquots for this pilot study were tested in a masked fashion within a routine batch of 2,000 specimens tested by the research laboratory (R.D.B.).

Statistical Analyses. χ² values, percent agreement, and percent positive agreement with 95% confidence intervals (95% CI) were calculated for the HPV DNA positivity of the 137 pooled specimens (observed) compared with the predicted HPV DNA testing results, based on the assumption that a single positive individual specimen included in a pool would result in the positive test for the pooled specimen (expected positive). The same statistics were used to evaluate the intra-batch reliability of the pooled specimens tested twice and the inter-batch reliability of tests on individual specimens. The McNemar’s χ² was used to evaluate whether the difference in positivity was significantly different (P < 0.05) for paired outcomes.

We then explored the relationship of viral genome concentration on test positivity of pooled specimens. Stratification was used to examine the concordance of observed and expected HPV DNA positivity in relation to number of interim visits per pooled specimen [categories of 2-3 (n = 61), 4-6 (n = 61), and 7-12 (n = 65) visits]. Next, we created surrogate variables of expected viral genome concentration by dividing the following by the total number of interim visits: (1) the number individual positive tests (estimated “positive tests/visit”), (2) the sum of general probe signal strength for each positive visit (estimated “general probe signal strength/visit”), and (3) the sum of type-specific signal strength for each type detected (estimated “type-specific signal strength/visit”). Receiver operator characteristic (ROC) analyses (12) were used to explore the relationships of these surrogates to the observed test positivity by calculating the area under the ROC curve and corresponding 95% CI (an area of 1 indicates that these

4 Women with evidence of possible cervical intraepithelial neoplasia grade 2, grade 3, or cancer by visual inspection, cytology, or Cervigram evaluation were referred to colposcopy, treated if necessary, and censored from follow-up.
Table 1. A comparison of the expected and observed HPV DNA test positivity

<table>
<thead>
<tr>
<th>Observed HPV status</th>
<th>No. negative</th>
<th>No. positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expected HPV status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. negative</td>
<td>110</td>
<td>6</td>
<td>116</td>
</tr>
<tr>
<td>HPV+</td>
<td>25</td>
<td>46</td>
<td>71</td>
</tr>
<tr>
<td>No. positive</td>
<td>35.2%</td>
<td>64.8%</td>
<td>100.0%</td>
</tr>
<tr>
<td>Total</td>
<td>135</td>
<td>52</td>
<td>187</td>
</tr>
</tbody>
</table>

NOTE: Row percentages are shown beneath the number in each cell. 

Results

As shown in Table 1, there was a moderate agreement between HPV DNA test positivity for the pooled specimens compared with the expected test positivity predicted by the test results for the individual specimens in the pool, with a $\kappa$ of 0.63 (95% CI, 0.51-0.75), a percent agreement of 83.4% (95% CI, 77.3-88.5%), and a percent positive agreement of 59.7% (47.9-70.8%). Overall, the observed HPV DNA positivity was less than the expected HPV DNA positivity ($P = 0.001$); 46 of 71 (64.8%) predicted to be HPV DNA-positive tested HPV DNA-positive. However, of the 46 expected and observed HPV DNA-positive pooled specimens, 6 specimens (13.0%) tested positive for HPV types that were completely different than predicted and 44 specimens (87.0%) had at least one type in common with the predicted types, of which 30 specimens (56.5%) had exactly the predicted types (Table 2).

In the subset of 83 pooled specimens with repeat testing, we observed a $\kappa$ of 0.68 (95% CI, 0.53-0.84), a percent agreement of 84.3% (95% CI, 74.7-91.4%), and a percent positive agreement of 75.9% (95% CI, 62.4-86.5%) for HPV DNA positivity. Of the 41 that tested positive for both pooled aliquots, 3 specimens (7.3%) tested positive for HPV types that were completely different and 38 specimens (92.7%) tested positive for at least one type in common, of which 32 specimens (78.0%) tested positive for exactly the same types.

In the subset of 66 retested individual specimens, there was a $\kappa$ of 0.82 (95% CI, 0.67-0.97), a percent agreement of 92.4% (95% CI, 85.2-97.5%), and a percent positive agreement of 77.3% (95% CI, 54.6-92.2%) compared with the original test result for HPV DNA positivity. There were no significant differences in HPV positivity between the original and second tests ($P = 0.2$). Of the 17 individual specimens that tested positive on both the original test and the retest, 3 specimens (13.6%) tested positive for different HPV types and 14 specimens (82.3%) tested positive for at least one type in common, of which 9 specimens (52.9%) tested positive for exactly the same types.

Stratified on number of interim visits, there was no difference in positivity between expected and observed for 2 to 3 visits [23.0% versus 23.0%, $P = 1.0$, McNemar’s $\chi^2$; $\kappa = 0.72$ (95% CI, 0.51-0.93); percent agreement = 90.2% (95% CI, 79.8-96.3%); percent positive agreement = 64.7% (95% CI, 38.3-85.8%)], a nonsignificant difference for 4 to 6 visits [39.3% versus 31.1%, $P = 0.1$, McNemar’s $\chi^2$; $\kappa = 0.68$ (95% CI, 0.49-0.87); percent agreement = 86.9% (95% CI, 75.8-94.2%); percent positive agreement = 69.2% (95% CI, 48.2-85.7%)], and a significant difference for 7 to 12 visits [50.8% versus 29.2%, $P = 0.001$, McNemar’s $\chi^2$; $\kappa = 0.51$ (95% CI, 0.30-0.72); percent agreement = 84.6% (95% CI, 73.4-92.4%); percent positive agreement = 69.7% (95% CI, 51.3-84.4%)].

Discussion

Recently, pooling of DNA samples to detect rare genetic variants has gotten attention (14-17). In this pilot study, we showed that pooling cervical cell specimens for PCR testing may be a useful method to discriminate women who have some HPV infections from those that do not, when multiple specimens need to be tested and HPV prevalence is expected to be low. We used this approach in a group of women who did not have overt disease or long-term viral persistence based on our testing of baseline and exit specimens. There was some loss in analytic sensitivity due to pooling, especially in pools of larger numbers of specimens, but this must be weighed against time, manpower, and the cost of testing of many specimens from women who would be expected to have entirely HPV DNA-negative sets of cervical specimens. This approach could be simplified further by performing the HPV PCR without typing the amplicons. Based on such a schema, rather than testing the 1,013 individual specimens that were included here, 187 pooled specimens would be screened by HPV PCR and the individual specimens of 52 pooled positives (n = 293 individual specimen tests and 480 total tests) could then be

Table 2. A comparison of the expected and observed type-specific HPV DNA test results

<table>
<thead>
<tr>
<th>Expected HPV status/observed HPV status</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV-/HPV-</td>
<td>110 (58.8)</td>
</tr>
<tr>
<td>HPV+/HPV-</td>
<td>25 (13.4)</td>
</tr>
<tr>
<td>HPV-/HPV+</td>
<td>6 (3.2)</td>
</tr>
<tr>
<td>HPV+/HPV+</td>
<td>14 (7.5)</td>
</tr>
<tr>
<td>[different type(s)]</td>
<td>6 (3.2)</td>
</tr>
<tr>
<td>[same type(s)]</td>
<td>26 (13.9)</td>
</tr>
<tr>
<td>Total</td>
<td>187 (100.0)</td>
</tr>
</tbody>
</table>
algorithm of pooling and testing by HPV PCR without typing the amplicons before typing individual specimens from positive pools, we crudely estimate that the absolute risk would be 2.1%.

There are several important additional caveats. First, some HPV types were missed in pooled specimens containing multiple HPV types and, therefore, using the type-specific results from pooled testing as a summary of HPV type-specific exposures without testing individual specimens comprising test-positive pooled specimens warrants caution. Only 30 of the 71 (42.2%) expected positives tested HPV DNA-positive for exactly the predicted types. This error was not appreciably different from the one obtained when we compared the results from retesting individual specimens with the previous test results (9 of 21, 42.9%) but both were lower than the fraction of identical types detected in the duplicate (intra-batch) test results for the pooled aliquots (32 of 47, 68.1%), suggesting that typing differences between expected and observed were the result of inherent interbatch variability and not necessarily the result of pooling specimens. The discordant type results were likely the result of low signal strength; the signal strength of pooled specimens that tested positive for type(s) different from the expected types was lower than for those that tested positive for at least one expected type ($P = 0.03$, Pearson $\chi^2$). Again, the trade-off of testing efficiency versus information may be acceptable when resources are limited. Second, the duration of these short-term infections in this subset of women cannot be estimated from pooling data and therefore any estimates of infection duration for the entire population of women will be overestimated. In this sample of women, almost all infections were detectable by PCR for only one visit (93.6%). Third, the concordance of the pooled test result with the history of exposure was dependent on the number of specimens pooled, with poorer concordance occurring among women with greater numbers of specimens collected and pooled. This phenomenon seems to be directly related to viral genome concentration, as the area under the curve in the ROC was greater with better surrogates of viral genome concentration. As expected, greater dilution due to greater number of specimens pooled leads to low genome concentrations and poorer reproducibility due to lower signal-to-noise ratios. Reconstruction experiments with samples containing known amounts of viral genomes are needed to confirm this explanation.

To minimize the effect of poorer sensitivity with larger numbers of specimens per woman, the number of specimens pooled could be limited such that for some extensive specimen sets, more than one pooled specimen is created. In this study, ≤5 interim specimens (42% of all women had five or less interim visits) might have been an ideal pooling size for the screening test, with a $k$ of 0.74 (95% CI, 0.57-0.91), percent agreement of 89.7% (95% CI, 80.8-95.5), and a percent positive agreement of 68.0% (95% CI, 46.5-85.1%). Based on these pilot data, limiting the pooling to five or less specimens would increase the sensitivity to 77.3% compared with the 65% for pooling all individual specimens; assuming that specimens with six or more specimens were divided into equal pools of five or less consecutively collected specimens, there would be a concomitant increase in the number of tests ($n = 268$ individual specimen tests and 565 total tests) compared with pooling all individual specimens.

We note that ~5% of specimens predicted to be negative tested positive. We cannot ascertain whether these few discordant results ($n = 6$) were falsely negative on the original test or falsely positive upon retesting of the pooled specimens. There is inherent variability in all assays and poorer reproducibility when the signal-to-noise ratio is low as is the case with testing of these specimens. A previous report on the intra-laboratory reliability for the same PCR assay found a

amplified and HPV typed for significantly greater testing efficiency. The sensitivity of testing pooled specimens would have been 65% compared with the expected positivity, and the negative predictive value is 81.5%. The estimated sensitivity for pooled testing of the entire group of women who were HPV DNA-negative at enrollment and at exit is 67%.

Consequences of the loss of sensitivity for these transient HPV infections include an underestimation of the number of women who were actually exposed to HPV and an overestimation of the absolute risk. For example, we crudely estimate that the absolute risk for CIN3 or cancer over 5 to 7 years given oncogenic HPV DNA positivity would be 1.6%; based on an

Figure 1. ROC analyses of observed HPV DNA positivity versus the calculated number of HPV DNA-positive specimens/visit (A), general probe signal strength/visit (B), and type-specific probe signal strength/visit (C).
similar retest positivity for negative specimens tested individually (not pooled; ref. 18).

We conclude that HPV DNA testing by PCR of a pooled specimen comprised of specimens from the same woman represents a trade-off in accuracy versus practicality. It may provide a practical screening method for identifying positive HPV specimens within sets of longitudinally collected specimens from women who have little in the way of overt disease or surrogates of disease such as prolonged viral persistence. This screen will reduce the number of cervical specimens to be tested for HPV DNA. This could be particularly valuable for large cohort studies like the one in Guanacaste, which, despite high prevalence of HPV and historically high levels of cancer, will have many women with intensive cervical sampling but no detectable HPV infections. The effectiveness of this approach using other primer systems, such as GP5+/6(+)(19) and SPF (10, 20), which amplify shorter amplicons and therefore may be more efficient for detecting low viral genome concentrations in specimen pools, deserves evaluation. This approach, PCR testing of pooled specimens, might also be useful in other large research studies involving multiple follow-up visits and the collection of multiple specimens with exposure measurements other than HPV.

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References

PCR Testing of Pooled Longitudinally Collected Cervical Specimens of Women to Increase the Efficiency of Studying Human Papillomavirus Infection

Philip E. Castle, Mark Schiffman, Rolando Herrero, et al.


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