Comparison of Linear Array and Line Blot Assay for Detection of Human Papillomavirus and Diagnosis of Cervical Precancer and Cancer in the Atypical Squamous Cell of Undetermined Significance and Low-Grade Squamous Intraepithelial Lesion Triage Study

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We evaluated Linear Array (LA), a newly commercialized PGMY09/11 L1 consensus primer PCR test that detects 37 human papillomavirus (HPV) genotypes by reverse line blot hybridization, for the detection of individual HPV genotypes and carcinogenic HPV and its clinical performance for detecting 2-year cumulative cervical precancer and cancer using archived specimens from the Atypical Squamous Cell of Undetermined Significance (ASCUS) and Low-Grade Squamous Intraepithelial Lesion Triage Study. LA testing was conducted on enrollment specimens from women referred because of an ASCUS Pap test. To gauge the performance of the new test, the results were compared to those of its prototype predecessor assay, Line Blot Assay (LBA), restricted to paired results (n = 3,335). LA testing was done masked to LBA results and clinical outcomes. The results of LA and LBA testing were compared for detection of carcinogenic HPV and clinical outcomes of cervical precancer and cancer. Overall, 50% and 55% of the women tested positive for carcinogenic HPV by LBA and LA, respectively (P < 0.0001). The percent agreement for carcinogenic HPV detection was 88%, percent positive agreement was 80%, and kappa was 0.76 for detection of carcinogenic HPV by the two assays. There was a significant increase in detection by LA for most of the 37 HPV genotypes targeted by both assays, including for 13 of 14 carcinogenic HPV genotypes. LA detected more multiple-genotype infections for all HPV genotypes among HPV-positive women (P < 0.0001) and for carcinogenic HPV genotypes among carcinogenic-HPV-positive women (P < 0.0001). LA was more sensitive (92.3% versus 87.1%; P = 0.003) and less specific (48.2% versus 54.0%; P < 0.0001) than LBA for 2-year cumulative cervical precursor and cancer as diagnosed by the Pathology Quality Control Group. In conclusion, we found LA to be a promising assay for the detection of HPV genotypes and carcinogenic HPV, and it may be clinically useful for the detection of cervical precursor and cancer in women with equivocal cytology.

It is now generally recognized and accepted that approximately 15 cancer-associated (carcinogenic) human papillomavirus (HPV) genotypes cause virtually all cervical cancer and its immediate precursor lesions, cervical precancer, diagnosed histopathologically as cervical intraepithelial neoplasia grade 3 (CIN3). HPV infections are common and typically resolve within 1 to 2 years. Uncommonly, carcinogenic HPV infections persist; such persistence is highly linked to the development of CIN3, which, if left untreated, can potentially invade.

Based on knowledge of the central role of persistent, carcinogenic HPV in cervical carcinogenesis, one pooled-probe test for carcinogenic genotypes of HPV DNA (as a group) has already been FDA approved (Hybrid Capture 2 [hc2]; Digene Corporation, Gaithersburg, MD), and other tests may soon be widely available. There are ample prospective data to suggest that a single baseline HPV DNA test is much more sensitive than the single conventional Pap test for the detection of CIN3 or cancer (CIN3 or worse) over 2 to 10 years (16, 17, 19, 44). Recent randomized trials have emphatically confirmed these findings (9, 36, 37). Also, one randomized trial demonstrated that HPV DNA testing is a safe triage of equivocal cytology (47). Accordingly, HPV testing has now been approved in the United States as an adjunct to cytology for triage at all ages and for general screening in women ≥30 years old (54). HPV DNA negativity implies an extremely low risk of prevalent or incident cancer/CIN3 (17, 27, 44), thereby safely permitting an extension of screening intervals.

Although tests to detect carcinogenic HPV have proven to be clinically useful for predicting cervical precursor and cancer, it is now apparent that there is important variability in the risk depending on the carcinogenic HPV genotype. For example, HPV type 16 (HPV16) causes 50% to 60% of all cervical cancer, and HPV18 causes another 15% to 20%, while other carcinogenic HPV genotypes contribute smaller proportions of cancers. Thus, even among carcinogenic-HPV-positive women, there may be value in the discrimination of individual HPV genotypes, particularly HPV16 and HPV18 (5, 8, 12, 26, 32, 51), to determine which women are at greater and lesser risk of...
cervical precancer and cancer. In international case series (45) and in population-based studies (24), HPV-45 is typically the next most important HPV type for causing cervical cancer. Other studies have found evidence that genotype-specific detection of HPV31 and HPV33 is useful for detection of cervical precancer and cancer risk stratification (5, 32). Current American Society of Colposcopy and Cervical Pathology guidelines for management of women with abnormal cervical cancer screening tests suggest that genotyping for HPV16 and HPV18 may be useful as a triage test for women who are cytologically negative and who test carcinogenic-HPV positive to determine who needs colposcopy, once an HPV-genotyping test is FDA approved (52).

Moreover, given the role of persistent carcinogenic-HPV infection in cervical carcinogenesis, there is good reason to believe that the detection of persistent infection will be a specific marker of risk that may be used clinically to distinguish persistent infections from transient infections that pose little risk. Several epidemiologic studies have demonstrated the very high relative and absolute risks of cervical precancer and cancer in the context of type-specific persisting carcinogenic-HPV infections (28, 40, 43). There is some evidence of increased clinical performance of HPV testing with an emphasis on 1-year viral persistence rather than one-time detection (30). If the risk stratification provided by HPV testing is improved by determining genotypes and measuring genotype-specific persistence, there may be clinical utility for genotype-specific HPV detection for distinguishing women at higher risk (persistent infection) from those at lower risk (sequential infections by different HPV types) of cervical precancer and cancer, once the validity of an HPV-genotyping assay(s) has been established (48, 49). Critical to the use of any HPV-genotyping test will be the establishment of rigorous quality control (33) and methods (1–3, 39, 47). The National Cancer Institute and local institutional review boards approved the study, and all participants provided written informed consent. This analysis was restricted to women referred for an ASCUS Pap test, because previous work had indicated that carcinogenic HPV testing was not clinically useful in young women with LSIL Pap results (1).

Epidemiologic studies have relied on several PCR-based assays for natural-history studies of HPV. These PCR assays have primarily utilized L1 consensus primer amplification with a pool of primers to coamplify all major anogenital HPV genotypes, followed by detection of specific HPV genotypes achieved by reverse hybridization via genotype-specific probes immobilized on a solid support. Commonly used primer sets for HPV genotyping have included GP5+/6+ (25), SPF10 (29), and MY09/11 (4), the last of which has gone through several modifications to improve its amplification of a broad range of HPV genotypes.

One of the improved versions of MY09/11, PGMY09/11 (22) was first developed into a prototype test, the Line Blot Assay (LBA) (Roche Molecular Systems, Alameda, CA) and has been extensively used in epidemiologic studies (20, 34, 35, 42, 50, 51). The LBA has now been commercialized as the Linear Array (LA) (Roche Molecular Systems), which is currently being evaluated in clinical trials and is CE marked for use in Europe (where CE marking represents conformity with health and safety requirements in directives created by the European Union). LA detects 37 HPV genotypes, including all known carcinogenic HPV genotypes. To provide an independent evaluation of LA, we tested the enrollment specimens from women with atypical squamous cell of undetermined significance (ASCUS) Pap results enrolled in the ASCUS Low-Grade Squamous Intraepithelial Lesion (LSIL) Triage Study (ALTS), a clinical trial to evaluate management strategies for women with equivocal or mildly abnormal Pap tests. To gauge the performance of the commercial assay kit, we compared the results from LA testing to those of its prototype assay, LBA, and to a rigorous endpoint of 2-year cumulative CIN3 or worse histology.

MATERIALS AND METHODS

Study design and population. ALTS was a randomized trial comparing three management strategies for 5,060 women with ASCUS (n = 3,488) or LSIL (n = 1,572) Pap results (39): (i) immediate colposcopy (immediate-colposcopy arm; referral to colposcopy regardless of enrollment test results), (ii) HPV triage (HPV arm; referral to colposcopy if the enrollment HPV result was positive by hc2 or missing or if the enrollment cytology was high-grade squamous intraepithelial lesion [HSIL]), or (iii) conservative management (conservative-manage- ment arm; referral to colposcopy if the enrollment cytology was HSIL). (We maintain the use of ASCUS, based on the 1991 Bethesda System for cytologic classification of cervical liquid-based cytologic interpretation.) At enrollment, all women received a pelvic examination with collection of two cervical specimens: the first specimen in PreservCyt for ThinPrep cytology (Cytec Corporation, Marlborough, MA) and hc2 testing and the second in specimen transport medium (STM) (Digene Corporation). The women in all three arms of the study were reevaluated by cytology every 6 months for 2 years of follow-up and were sent to colposcopy if the cytology was HSIL. An exit examination with colposcopy was scheduled for all of the women, regardless of the study arm or prior procedures, at the completion of the follow-up. We refer readers to other references for details on randomization, examination procedures, patient management, and laboratory and pathology methods (1–3, 39, 47). The National Cancer Institute and local institutional review boards approved the study, and all participants provided written informed consent. This analysis was restricted to women referred for an ASCUS Pap test, because previous work had indicated that carcinogenic HPV testing was not clinically useful in young women with LSIL Pap results (1).

HPV DNA testing. HPV genotyping by LBA, an L1 consensus primer-based PCR assay that employs a primer set designated PGMY09/11, was performed on the STM specimen as previously described (22). The amplicons were subjected to reverse line blot hybridization for detection of 27 individual HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51 to 59, 66, 68, 73 [PAP238a], 82 [W13b], 83 [Pap291], and 84 [PAP155]) (23). During the enrollment phase of testing, the capacity of the reverse line blot strip to detect individual HPV genotypes was expanded from 27 HPV genotypes to 38 HPV genotypes to include detection of an additional 11 noncarcinogenic HPV genotypes (61, 62, 64, 67, 69 to 72, 81, 82 variant [82v or IS39], and 89 [CP6108]). Thus, 76% of the enrollment specimens from women referred because of an ASCUS Pap test were tested for 38 HPV genotypes. An equivalent 1.5% of the original specimen was used for LBA (42).

Aliquots of the archived STM specimens from women referred because of an ASCUS Pap test were also tested using LA, a commercial version of LBA (although still for research use only in the United States) that tests for 37 of 38 HPV genotypes included in the LBA, excluding only the rare, noncarcinogenic HPV57. Specimens were tested by LA a median of 104 months after collection and 54 months after being tested by LBA. Because of intellectual property rights, LA does not directly detect HPV52 but combines a set of probes that detects HPV33, -35, -52, and -58 combined (HPVmix). Specimens that test negative for HPV33, -35, and -58 individually but are positive for HPV51 are considered to be HPV52 positive. The specimens that test positive for HPV51 and for HPV33, -35, and/or -58 have an uncertain HPV52 status, and for this analysis, these specimens were considered to be HPV52 negative. LA was used according to the manufacturer’s instructions in the product insert, which include DNA extraction using the QIAamp MinElute Media Kit (Qiagen, Inc., Valencia, CA). The only deviation from the LA product insert protocol was to implement an automated sample preparation for extraction of up to 96 specimens at a time on the QiaGen Mdx platform (using the MinElute Media Mdx Kit and the manufacturer’s...
instructions) rather than processing 24 specimens per batch by the manual vacuum method (10). This method of automatic sample preparation is now being evaluated in conjunction with clinical trials for the validation of LA. An equivalent of 2.8% of the original specimen was used for LA.

After the preparation of liquid-based cytology, 4-ml aliquots of residual PrecySt-Cyt specimens were tested by hc2 (47), a pooled-probe, signal amplification DNA test that targets a group of 13 HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) (41) and cross-reacts with a 14th carcinogenic HPV genotype, HPV53 (11, 38, 42). hc2 signal strength (in relative light units per positive control RLU/pc) was considered a semiquantitative measure of the HPV load ("HPV semiquantitative load") (21).

HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 were considered the primary carcinogenic genotypes (6, 13). For the detection of individual HPV genotypes and any carcinogenic HPV by the prototype assay, HPV16, HPV18, or any of 14 carcinogenic HPV genotypes by the commercial assay, LA and LBA, respectively (47, 52, 56, 58, 59, 68) (41) and cross-reacts with a 14th carcinogenic HPV genotype, HPV53 (11, 38, 42). hc2 signal strength (in relative light units per positive control RLU/pc) was considered a semiquantitative measure of the HPV load ("HPV semiquantitative load") (21).

HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 were considered the primary carcinogenic genotypes (6, 13). For the results of each HPV-genotyping test, women were assigned to a priori-established HPV risk groups according to cervical cancer risk: group 1, positive for HPV16; group 2, positive for HPV18; group 3, positive for any carcinogenic HPV genotype and negative for HPV16 and HPV18 (carcinogenic HPV excluding HPV16 and HPV18); group 4, positive for any noncarcinogenic HPV genotypes and negative for all carcinogenic HPV genotypes; or group 5, PCR negative (group ranking according to risk is as follows: HPV16 > HPV18 > carcinogenic HPV excluding HPV16 and HPV18 > noncarcinogenic HPV > PCR negative).

References to LBA or LA refer to the entire method used, which included specimen preparation, extraction, amplification, and genotyping.

**Pathology and treatment.** Clinical management was based on the clinical-center pathologist's cytologic and histologic diagnoses. In addition, all referral smears, ThinPreps, and histology slides were sent to the Pathology Quality Control Group (QC pathology) based at the Johns Hopkins Hospital for independent review and diagnosis. CIN2 or worse diagnosis based on the clinical-center pathology or a CIN3 or worse diagnosis based on the QC pathology review triggered treatment by loop electrosurgical excision procedure. In addition, women with persistent LSIL or carcinogenic HPV-positive AS-CUS at the time of exit from the study were offered the loop electrosurgical excision procedure.

**Statistical analysis.** Detection of individual HPV genotypes and any carcinogenic HPV genotype, the distribution of the number of genotypes (0, 1, 2, 3, 4, and 5 +) (considering only common HPV genotypes tested for by both assays, 2b or 37, on a given specimen) and carcinogenic HPV genotypes detected, and categorization of women according to HPV risk groups by LBA and LA were compared by calculating kappa values, percent agreement, and percent positive agreement. Differences in detection were tested for statistical significance ($P < 0.05$) using an exact McNemar's $\chi^2$ (2 categories) or symmetry $\chi^2$ ($>2$ categories). The results were stratified on hc2 status (negative or positive), log units of the HPV semiquantitative load (1.00 to 9.99, 10.00 to 99.99, 99.99 to 999.99, and $\geq 1.000$ RLU/pc), and enrollment cytology (negative versus hc2 positive and cytology positive; alternatively, negative versus ASC-US, and LSIL versus HSIL/ASC-H).

The specificity, sensitivity, and PPV and NPV of LA- and LBA-detected carcinogenic HPV were calculated for histologically confirmed CIN3 or worse as diagnosed by QC pathology and histologically confirmed CIN2 or worse as diagnosed by the clinical-center pathologists over the 2-year duration of ALTS. For the latter endpoint, the analysis was restricted to the immediate-colposcopy and HPV arms of the trial because of the evidence of regression of CIN2 in the conservative-management arm of the trial (2, 3). Differences in test sensitivity and specificity for CIN3 or worse and for CIN2 or worse were evaluated for statistical significance using an exact McNemar's $\chi^2$. Differences in PPV and NPV were tested for statistical significance using a method developed by Leisenring et al. (31), a score statistic derived from a marginal regression model that bears some relation to McNemar's $\chi^2$. Differences in PPV and NPV were tested for statistical significance using a method developed by Leisenring et al. (31), a score statistic derived from a marginal regression model that bears some relation to McNemar's $\chi^2$. Differences in PPV and NPV were tested for statistical significance using a method developed by Leisenring et al. (31), a score statistic derived from a marginal regression model that bears some relation to McNemar's $\chi^2$. Differences in PPV and NPV were tested for statistical significance using a method developed by Leisenring et al. (31), a score statistic derived from a marginal regression model that bears some relation to McNemar's $\chi^2$.

**RESULTS**

**Carcinogenic-HPV detection.** Table 1 shows the detection of any of 14 carcinogenic HPV genotypes by the prototype assay, LBA, and the commercial version, LA, for women referred to ALTS for an ASCUS Pap smear. Overall, there were 3,335 test results for LBA (95.6%), 3,446 test results for LA (98.8%), and 3,335 paired test results (95.6%). Among those with paired results, 50% and 55% of the women tested positive for carcinogenic HPV by LBA and LA, respectively ($P < 0.0001$). The percent agreement was 88%, percent positive agreement was 80%, and kappa was 0.76 for detection of carcinogenic HPV by the two assays.

Of women with normal enrollment cytology, 30% tested positive for carcinogenic HPV by LBA compared to 38% by LA ($P < 0.0001$). Of women with ASC-US enrollment cytology, 50% tested positive for carcinogenic HPV by LBA com-
Differences were tested for statistical significance using an exact McNemar's test. Genotypes targeted by both assays, with the notable exception of carcinogenic HPV52, which was more commonly detected by LA (Table 2). There was also no difference in detection of typically rare genotypes, such as HPV64 and HPV69, and rare genotypes in this population, such as 71 and 82v. The relative increase (as a ratio of the prevalence of individual HPV genotypes measured by LA versus LBA [Table 2]) in the detection of carcinogenic HPV genotypes by LA compared to LBA, except for HPV52, ranged from 11% (HPV33) to 47% (HPV70). Among women who tested negative by hc2, 11% tested positive for carcinogenic HPV by LBA compared to 20% by LA (P < 0.0001). Among women who tested positive by hc2, 84% tested positive for carcinogenic HPV by LBA compared to 87% by LA (P < 0.0001); the percent agreement increased from 84% to 96% and the positive percent agreement increased from 79% to 96% for increasing log units of HPV semiquantitative load.

Genotype-specific HPV detection. There was an increased detection by LA compared to LBA for most of the 37 HPV genotypes targeted by both assays, with the notable exception of carcinogenic HPV52, which was more commonly detected by LA (Table 2). There was also no difference in detection of typically rare genotypes, such as HPV64 and HPV69, and rare genotypes in this population, such as 71 and 82v. The relative increase (as a ratio of the prevalence of individual HPV genotypes measured by LA versus LBA [Table 2]) in the detection of carcinogenic HPV genotypes by LA compared to LBA, except for HPV52, ranged from 11% (HPV33) to 47% (HPV70). The prevalence of HPV genotypes, as expected, was lower in subgroups of hc2-negative and cytology-negative women than in subgroups of hc2-positive and cytology-positive women, respectively. Increases in detection by LA versus LBA were generally greater in the hc2-negative and cytology-negative women than in subgroups of hc2-positive and cytology-positive women, respectively. The increased detection of individual genotypes by LA compared to LBA was most pronounced for the 37 HPV genotypes targeted by both assays, with the notable exception of carcinogenic HPV52, which was more commonly detected by LA (Table 2).
pared with LBA is reflected in the greater number of multigeno-
type HPV infections and the greater number of genotypes per
multigenotype HPV infection, both for all HPV genotypes (P <
0.0001) (Table 3) and for carcinogenic HPV genotypes (P <
0.0001) (data not shown). This finding remained when women
who had one or more HPV genotypes as detected by both assays
(P < 0.0001 for any HPV type and any carcinogenic HPV type)
and women who had two or more HPV genotypes detected by
both assays (P < 0.0001 for any HPV type and P = 0.0001 for
carcinogenic HPV types) were considered. The increase in the
number of all genotypes and carcinogenic HPV genotypes by LA
over LBA remained significant among hc2-negative and -positive
women and among women with normal and nonnormal cytolo-
gies (data not shown).

When the results of HPV genotype-specific testing were
stratified according to HPV risk groups, LA was more likely to
categorize women in a higher risk group than LBA (P <
0.0001) because of the greater detection of individual HPV
types that were tested for in all specimens by both assays.

Clinical performance. The clinical performances of the two
tests were evaluated versus two different endpoints, 2-year
cumulative CIN3 or worse histology diagnosed by QC pathol-
ogy as our most rigorous definition of a precursor and 2-year
cumulative CIN2 or worse histology diagnosed by clinical-cen-
ter pathologists, which was the treatment threshold for ALTS
and for standard clinical practice (Table 5). LA was more sen-
sitive (92.3% versus 87.1%; P = 0.003) and less specific (48.2%
versus 54.0%; P < 0.0001) than LBA for QC pathology-diagnosed CIN3 or worse. Similarly, LA was more sensi-
tive (87.5% versus 84.3%; P = 0.006) and less specific (50.7%
versus 56.6%; P < 0.0001) than LBA for clinical-center pa-
thologist-diagnosed CIN2 or worse. Consequently, LA had sig-
nificantly lower PPV (2-year cumulative risk) but greater NPV
(reassurance) than LBA.

The 2-year cumulative absolute risks of CIN3 or worse for
women according to the HPV risk groups as defined by either
assay were then evaluated and compared (Table 6). Three
definitions of CIN3 or worse were used: histological diagnosis
by QC pathology, by clinical-center pathologists, and by either
pathology review. For HPV risk groups as determined by LBA,
the risks of CIN3 or worse were 27.3% to 35.2% for HPV16-
positive women, 6.2% to 10.1% for HPV18-positive women,
7.6% to 10.2% for carcinogenic-HPV-positive women (exclud-
ing HPV16 positives and HPV18 positives), 2.0% to 3.7% for
noncarcinogenic-HPV-positive women, and 0.7% to 2.1% for

### Table 3. Comparison of the distribution of the numbers of HPV genotypes detected by LA versus LBA, restricted to the 26 HPV genotypes that were tested for in all specimens by both assays

<table>
<thead>
<tr>
<th>No. of HPV genotypes detected by LBA</th>
<th>No. (%) of specimens in which no. of HPV genotypes detected by LA was:</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>941 (28.2)</td>
<td>212 (6.4)</td>
</tr>
<tr>
<td>1</td>
<td>69 (2.1)</td>
<td>540 (16.2)</td>
</tr>
<tr>
<td>2</td>
<td>16 (0.5)</td>
<td>62 (1.9)</td>
</tr>
<tr>
<td>3</td>
<td>7 (0.2)</td>
<td>12 (0.4)</td>
</tr>
<tr>
<td>4</td>
<td>2 (0.1)</td>
<td>1 (0.0)</td>
</tr>
<tr>
<td>5+</td>
<td>6 (0.2)</td>
<td>5 (0.1)</td>
</tr>
<tr>
<td>Total</td>
<td>1,041 (31.2)</td>
<td>832 (24.9)</td>
</tr>
</tbody>
</table>

*Cells with boldface type indicate agreement for the HPV risk group. For all categories, P was <0.0001; symmetry χ²; Pₜₚₑᵣₒₑ = <0.0001; kappa was 0.53; percent total agreement was 64.0%. Restricted to HPV positive by both assays, P was <0.0001; symmetry χ²; Pₜₚₑᵣₒₑ = <0.0001; kappa was 0.48; percent total agreement was 61.0%.

### Table 4. Comparison of HPV risk groups

<table>
<thead>
<tr>
<th>HPV risk group by LBA</th>
<th>No. (%) of specimens for which HPV risk group by LA was:</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Noncarcinogenic</td>
</tr>
<tr>
<td>Negative</td>
<td>941 (28.2)</td>
<td>134^a (4.0)</td>
</tr>
<tr>
<td>Noncarcinogenic</td>
<td>31 (0.9)</td>
<td>282 (8.5)</td>
</tr>
<tr>
<td>Carcinogenic</td>
<td>44 (1.3)</td>
<td>26 (0.8)</td>
</tr>
<tr>
<td>HPV18</td>
<td>7 (0.2)</td>
<td>2 (0.1)</td>
</tr>
<tr>
<td>HPV16</td>
<td>495 (14.8)</td>
<td>562 (16.8)</td>
</tr>
</tbody>
</table>

^a HPV16 > HPV18 > carcinogenic HPV excluding HPV16 and HPV18 (Carcinogenic) > non-carcinogenic HPV (Noncarcinogenic) > PCR negative (Negative) as detected by LBA and LBA. Cells with boldface type indicate agreement for the HPV risk group. For all categories, P was <0.0001; symmetry χ²; Pₜₚₑᵣₒₑ = <0.0001; kappa was 0.72; percent agreement was 79.2%. Restricted to HPV positive by both assays, P was <0.0001; symmetry χ²; Pₜₚₑᵣₒₑ = <0.0001; kappa was 0.83; percent agreement was 89.0%.

^b Cells that contributed ≥10% to the χ² statistic for all categories.

^c Cells that contributed ≥10% to the χ² statistic for HPV positive by both assays.
LBA-negative women. For LA, the risks were 25.1% to 32.4% for HPV16-positive women, 6.2% to 10.5% for HPV18-positive women, 7.1% to 9.8% for carcinogenic-HPV-positive women (excluding HPV16 positives and HPV18 positives), 1.2% to 2.6% for noncarcinogenic-HPV-positive women, and 0.2% to 1.2% for LA-negative women.

DISCUSSION

We evaluated the analytic and clinical performance of LA, a commercial version of LBA that has been evaluated extensively in this population (12, 42). We found that LA was more analytically sensitive than LBA, resulting in greater detection of individual genotypes, as well as an increased detection of multigenotype infections and number of genotypes per multigenotype infection, all of which translated into a more clinically sensitive but less specific test for CIN3 or worse in a population of women referred to ALTS because of an ASCUS Pap test. The increase in analytic sensitivity in this larger study confirms an earlier comparison of LBA and LA in a smaller set of anogenital clinical samples (anal specimens, physician-collected cervical specimens, and self-collected cervicovaginal specimens) (15). This increase in analytic and clinical sensitivity in LA versus LBA resulted in a slightly less predictive, but more reassuring, commercial test, i.e., more women without disease were called positive (lower specificity), increasing the size of the denominator of women identified as “at risk,” and more women with disease were called positive, reducing the number of false-negative cases, respectively.

Increased detection of HPV by LA appears to be the result of true detection of HPV rather than analytic false positives. First, the detection of HPV types by LA among LBA negatives was strongly related to the number of sexual partners within the last year, as detection increased from 15% in women with 114 CASTLE ET AL. J. CLIN. MICROBIOL.

TABLE 5. Clinical performance of carcinogenic HPV detection by LA and LBA for 2-year cumulative CIN3 or worse as diagnosed by QC pathology and CIN2 or more as diagnosed by clinical center pathologists

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LA</th>
<th></th>
<th></th>
<th>LBA</th>
<th></th>
<th></th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Estimate (%)</td>
<td>95% CI (%)</td>
<td></td>
<td>Estimate (%)</td>
<td>95% CI (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QC pathology CIN3 or worse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>92.3</td>
<td>88.6–95.1</td>
<td></td>
<td>87.1</td>
<td>82.7–90.8</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>48.2</td>
<td>46.4–50.0</td>
<td></td>
<td>54.0</td>
<td>52.2–55.8</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>PPV</td>
<td>14.4</td>
<td>12.8–16.1</td>
<td></td>
<td>15.1</td>
<td>13.4–17.0</td>
<td>0.0004</td>
<td></td>
</tr>
<tr>
<td>NPV</td>
<td>98.52</td>
<td>97.77–99.07</td>
<td></td>
<td>97.90</td>
<td>96.98–98.45</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Referral</td>
<td>55.3</td>
<td>53.6–57.0</td>
<td></td>
<td>49.5</td>
<td>47.8–51.2</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Clinical-center pathology CIN2 or worse b</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>87.5</td>
<td>83.5–90.8</td>
<td></td>
<td>84.3</td>
<td>80.0–88.0</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>50.7</td>
<td>48.4–53.0</td>
<td></td>
<td>56.6</td>
<td>54.3–58.8</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>PPV</td>
<td>24.6</td>
<td>22.2–27.1</td>
<td></td>
<td>26.2</td>
<td>23.7–28.9</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>NPV</td>
<td>95.67</td>
<td>94.22–96.85</td>
<td></td>
<td>95.16</td>
<td>93.73–96.34</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Referral</td>
<td>55.2</td>
<td>53.1–57.3</td>
<td></td>
<td>49.8</td>
<td>47.7–51.9</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

a Differences in sensitivity, specificity, and referral were tested for statistical significance using an exact McNemar’s χ² test. Statistical differences in PPV and NPV were tested for statistical significance using a method developed by Leisenring et al. (31), a score statistic derived from a marginal regression model that bears some relation to McNemar’s statistic.

b Included only the 2,225 women in the immediate-colposcopy and HPV study arms of ALTS.

TABLE 6. Two-year cumulative absolute risk of histologically confirmed CIN3 or worse

<table>
<thead>
<tr>
<th>Parameter</th>
<th>n</th>
<th>QC pathology</th>
<th>CC pathology</th>
<th>Worst pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. CIN3 or more</td>
<td>% CIN3 or more</td>
<td>No. CIN3 or more</td>
<td>% CIN3 or more</td>
</tr>
<tr>
<td>LA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>1,041</td>
<td>11</td>
<td>1.1</td>
<td>2</td>
</tr>
<tr>
<td>Noncarcinogenic</td>
<td>450</td>
<td>11</td>
<td>2.4</td>
<td>6</td>
</tr>
<tr>
<td>Carcinogenic</td>
<td>1,120</td>
<td>86</td>
<td>7.7</td>
<td>79</td>
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<tr>
<td>HPV18</td>
<td>162</td>
<td>14</td>
<td>8.6</td>
<td>10</td>
</tr>
<tr>
<td>HPV16</td>
<td>562</td>
<td>165</td>
<td>29.4</td>
<td>141</td>
</tr>
<tr>
<td>LBA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>1,281</td>
<td>24</td>
<td>1.9</td>
<td>9</td>
</tr>
<tr>
<td>Noncarcinogenic</td>
<td>402</td>
<td>13</td>
<td>3.2</td>
<td>8</td>
</tr>
<tr>
<td>Carcinogenic</td>
<td>1,028</td>
<td>81</td>
<td>7.9</td>
<td>78</td>
</tr>
<tr>
<td>HPV18</td>
<td>129</td>
<td>11</td>
<td>8.5</td>
<td>8</td>
</tr>
<tr>
<td>HPV16</td>
<td>495</td>
<td>158</td>
<td>31.9</td>
<td>135</td>
</tr>
<tr>
<td>Total</td>
<td>3,335</td>
<td>287</td>
<td>8.6</td>
<td>238</td>
</tr>
</tbody>
</table>

a As diagnosed by QC pathology, clinical-center pathologists (CC pathology), or either pathology review (Worst pathology) for detection of HPV categorized as HPV risk groups by LA or by LBA.
zero or one recent partners to 24% in those with two or three recent partners and 33% in those with four or more recent partners (P < 0.0001) (data not shown). Second, increased detection of carcinogenic HPV by LA was correlated with increased detection by hc2, with 37% of LBA-negative/LA-positive women testing positive by hc2 compared to 13% of LBA-negative/LA-negative women (data not shown).

The primer and probe sequences and manufacturer, as well as master mixture formulations, are identical between the two assays. Development of the LA for commercialization has been reported to include optimization of thermal-cycling conditions relative to the ABI TC9700, optimization of β-globin coamplification, and standardization of quality control procedures in reagent manufacture (15), which likely resulted in a general improvement in assay performance. It is also important to emphasize that we compared assay methods rather than doing a direct comparison of the reagents. Specifically, there were notable differences in the sample preparation procedures used for the two assays that may have contributed to the differences in HPV detection observed, namely, the extraction method (a manual, centrifugation-based method using ethanol precipitation of a crude clinical-specimen digest using proteinase K for LBA versus a robotic, nucleic acid-based purification for LA) and the amounts of specimen used in the assay (1.5% for LBA [42] versus 2.8% for LA). Other pilot experiments in our laboratory comparing the LBA and LA reagents using equal amounts of DNA from the same extraction showed no difference in overall or HPV genotype-specific detection between LBA and LA, where as the input of increased amounts of DNA led to increased detection of HPV genotypes (data not shown), suggesting that a large fraction of the disagreement observed here is attributable to loss in detection at low copy numbers when a smaller amount of DNA is used for amplification in LBA PCR. While this explanation is not consistent with a previous report from Coutlee et al. (15), it is important to note that the two studies may not be directly comparable because (i) the majority of samples in this study were anal, not cervical; (ii) sample preparation was completely different from the standardized method for both LA and LBA; and (iii) many of the specimens were collected from HIV-positive patients, known to have higher viral copy numbers. Our explanation of DNA input for differences in performance between LA and LBA in this study is consistent with our observation that there is a greater difference in testing positive between LA and LBA in women with lower semiquantitative viral loads (versus higher viral loads) and those with normal cytology (versus nonnormal cytology) and that the median semiquantitative viral load for testing positive for a single carcinogenic HPV type was lower for LA than for LBA (22 RLU/pc versus 45 RLU/pc) (data not shown).

A robotic system for DNA extraction was employed in this study to handle the large number of tests. For less expert laboratories, robotic extraction may help standardize DNA extraction, resulting in more consistent extraction of specimens and test performance and enhanced reproducibility. However, it is unclear whether smaller, less expert laboratories will be able to afford robotics for DNA extraction and therefore will by necessity rely on manual extraction methods, which may increase test variability and consequently lower test performance (18). Additionally, validity of the extraction measures relies on full compliance with recommended protocols (quality assurance) and routine monitoring of test performance (quality control). Substandard quality assurance/quality control measures in a clinical setting will inevitably decrease test performance characteristics.

One limitation of LA is the complexity in detection of HPV52, which, because of patent rights, cannot be directly targeted by reverse line blot hybridization on the membrane. A pool of mixed probes that detect HPV33, -35, and -58, as well as HPV52, is used, and those specimens that test negative for the other three HPV genotypes individually but positive for the pooled probes are inferred to be HPV52 positive. Although somewhat cumbersome, this indirect detection of HPV52 by LA was in fair agreement with direct detection by LBA, with a kappa value of 0.63, a percent agreement of 94%, and a percent positive agreement of 50% (data not shown). However, the HPV52 status cannot be discerned if women test positive for one or more of the other HPV genotypes included in the pooled probes individually, as well as for the mixed probe. For assessing HPV persistence in a clinical setting, it will likely be necessary to consider all women positive for the mixed probe to be HPV52 positive to maximize the detection of persistent HPV52 and thereby maximize the safety of patients at the cost of falsely categorizing some women as having persistent carcinogenic HPV. An alternative method might be to use an independent HPV52-specific PCR method to confirm all mixed-probe-positive results. Coutlee et al. (14), using an HPV52-specific TaqMan PCR assay, confirmed the presence of HPV 52 in 63/99 (64%) specimens coinfected with HPV33, -35, or -58. Because the samples in that study were derived from HIV-positive men and women, known to have an increased rate of multiple HPV infection, this may be an upper limit of the relative misclassification that would result without independent HPV52 confirmation. Whether an additional HPV52-specific test is clinically useful for clarifying these genotype-indeterminate HPV infections detected by LA will depend on the cost, the ease of use, and the proportion of these potentially mixed infections that are HPV52 positive.

Theoretically, HPV genotyping may have clinical utility by identifying which carcinogenic HPV-positive women are at the greatest risk of having or developing cervical precursor and cancer. There is already evidence of the possible utility of identifying women who are HPV16 or HPV18 positive among cytologically negative, carcinogenic-HPV-positive women once an HPV-genotyping test is FDA approved (26, 52). HPV genotyping might also be used to monitor HPV infections for viral clearance or persistence, which is strongly linked to the development of precursor and cancer (53). However, the ultimate utility of any HPV-genotyping test for screening and risk assessment will depend not only on its clinical performance, but also on the cost of the test and its cost-effectiveness, as well as rational algorithms for its use. However, given the likely costs and logistics of its use, the value of HPV genotyping will be limited to developed countries with large health care expenditures.

In conclusion, we utilized archival ALTS specimens to conduct a rigorous evaluation of LA, including comparing our results to a previous benchmark for HPV genotyping, LBA, and to 2-year cumulative disease defined by pathology review. We found LA to be a clinically sensitive test for CIN3 or worse
in a population of women referred to ALTS because of an ASCUS Pap test and therefore potentially useful as a triage test to determine who is referred to colposcopy. There was a notable increase in analytic sensitivity for LA compared to its prototype, LBA, leading to a concomitant trade-off of increased clinical sensitivity with decreased specificity. There is increasing evidence that identification of specific HPV genotypes may be useful in screening for cervical cancer. HPV16- and/or HPV18-infected women with normal cytology have an elevated risk of cervical precancer and cancer that is greater than the risk for women infected with other carcinogenic HPV genotypes and above the risk for women with LSIL cytology (8, 26). Women with long-term-persistent HPV infections, especially by HPV16, have very high absolute risks of cervical precancer and cancer (40). Even detection of shorter-term HPV persistence over a year may increase the specificity of precancer and cancer (30). Therefore, an HPV-genotyping test such as LA will be useful for genotype-specific risk stratification. However, future studies will need to demonstrate reliable, consistent detection of persistent carcinogenic HPV to validate its clinical utility for monitoring persistent HPV infections leading to the detection of incident CIN3 or worse.

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