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Performance of the Aptima High-Risk Human Papillomavirus mRNA Assay in a Referral Population in Comparison with Hybrid Capture 2 and Cytology[▽]

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This study compared the Aptima human papillomavirus (HPV) (AHPV; Gen-Probe Incorporated) assay, which detects E6/E7 mRNA from 14 high-risk types, the Hybrid Capture 2 HPV DNA (HC2; Qiagen Incorporated) test, and repeat cytology for their ability to detect high-grade cervical lesions (cervical intraepithelial neoplasia grade 2+ [CIN2+]) in women referred to colposcopy due to an abnormal Papanicolaou (Pap) smear. A total of 424 clinical specimens, stored in liquid-based cytology (LBC) vials at room temperature for up to 3 years, were tested by repeat cytology, the AHPV assay, and the HC2 test. Assay results were compared to each other and to histology results. The overall agreement between the AHPV assay and the HC2 test was 88.4%. The sensitivity (specificity) of cytology, the HC2 test, and the AHPV assay for the detection of CIN2+ was 84.9% (66.3%), 91.3% (61.0%), and 91.7% (75.0%) and for the detection of CIN3+ was 93.9% (54.4%), 95.7% (46.0%), and 98.2% (56.3%), respectively. Of the disease-positive specimens containing high-risk HPV (HR HPV) DNA as determined by Linear Array (Roche Diagnostics), the AHPV assay missed 3 CIN2 and 1 microfocal CIN3 specimen, while the HC2 test missed 6 CIN2, 4 CIN3, and 1 cervical carcinoma specimen. The AHPV assay had a sensitivity similar to but a specificity significantly higher ($P < 0.0001$) than the HC2 test for the detection of CIN2+. The AHPV assay was significantly more sensitive ($P = 0.0041$) and significantly more specific ($P = 0.0163$) than cytology for the detection of disease (CIN2+).

Cancer of the cervix uteri has an incidence of 8.2 per 100,000 women per year in the United States, with a mortality of 2.5 per 100,000 women per year (21, 20).

In the past 3 decades, implementation of the Papanicolaou (Pap) test (cervical cytology) has significantly reduced mortality due to cervical cancer when utilized in organized screening programs. However, the Pap test has a relatively low sensitivity (51 to 74% depending on the study) for detecting cervical dysplasia in women presenting for routine screening, has limited reproducibility, and often yields equivocal results (1, 23). There is now overwhelming evidence that cervical cancer is caused by persistent human papillomavirus (HPV) infections with certain types of high-risk HPV (HR HPV) (20). However, simply detecting an HPV infection provides only modest clinical benefit, as many HPV infections and precancerous lesions resolve without treatment. Thus, the American Society for Colposcopy and Cervical Pathology has recommended HPV testing as an adjunct to cervical cytology for screening for women 30 years of age and older (29). According to its guidelines, women with atypical squamous cells of undetermined significance (ASC-US) cytology and a positive HR HPV test should be referred directly to colposcopy (29). Indeed, combining cytology and HPV testing has been shown to yield a higher sensitivity than either test alone (7, 16), to increase the negative predictive value to 99 to 100% (7), and to reduce the incidence of medium- and high-grade cervical intraepithelial neoplasia (CIN) and cervical cancer (22).

Currently, only two commercially available HPV tests are approved by the United States Food and Drug Administration (FDA) for detection of HPV DNA in clinical specimens: the Hybrid Capture 2 (HC2) HPV DNA test (Qiagen, Hilden, Germany) and the Hologic Cervista high-risk HPV DNA test (Hologic Incorporated, Bedford, MA). Another test, the Roche Amplicor HPV DNA test, was introduced in the European Union in 2004. Tests based on HPV DNA detect the presence of high-risk HPV DNA, regardless of whether the infection is transient or persistent and regardless of disease severity.

The Gen-Probe Aptima HPV (AHPV) assay uses a different approach that consists of detecting mRNA from high-risk HPV E6/E7 oncogenes. This design approach is based on the finding that expression of HPV E6 and E7 mRNA increases with the increasing severity of cervical disease (2, 6, 8, 24). The possible benefit of detecting these transcripts is the enhanced ability to differentiate between transient HPV infections (which do not cause disease) and persistent infections (which are more likely to induce cellular abnormalities). Another important benefit includes improved specificity for the detection of high-grade cervical lesions (CIN2+), i.e., CIN2, CIN3, and cervical cancer (6, 28). Thus, Aptima HPV mRNA testing may reduce the patient burden and health care-related cost associated with colposcopy.

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The present study is aimed at comparing cytology with the performance of the AHPV assay and the HC2 test in detecting cervical disease in women referred to colposcopy due to an abnormal Pap smear.

MATERIALS AND METHODS

Specimens. Between February 2005 and May 2008, 492 liquid-based cytology (LBC) and conventional cytology specimens were collected from 451 women who were referred to the Universitaets-Frauenklinik Freiburg (Germany) for colposcopy due to an abnormal Pap screening result or for follow-up visits after therapy. After conventional cytology and LBC were performed, the residual LBC specimens were stored at room temperature for up to 3 years prior to HPV testing with both an AHPV assay and an HC2 test.

Sixty-eight specimens from 68 women were excluded from the analysis of results and included 32 specimens that did not yield a visible cell pellet after centrifugation and yielded a negative HC2 result, 11 specimens with vaginal or vulval dysplasia (or carcinoma), 9 specimens from pregnant women with positive cytology that were not subjected to biopsy, and 16 women with positive cytology and no available histology.

The remaining 424 cytology specimens from 385 women were used for the analysis of results. From 39 women, more than one specimen was collected (for 5 women 3 specimens were collected): at the first referral and at the follow-up visit(s) after therapy for CIN lesions.

The study was evaluated and approved by the local ethics committee.

Colposcopy and diagnostic procedures. Colposcopy was performed by the same physician (the corresponding author) to ensure consistent evaluations. Colposcopy findings were documented using a digital camera. During colposcopy (before staining with acetic acid and iodine), 3 types of specimens were collected in the following order: broom for LBC, ectocervical spatula, and endocervical cytobrush for conventional cytology. For LBC specimens, exfoliated cervical cells were collected into a ThinPrep Pap test vial containing PreservCyt solution (ThinPrep Pap test; Hologic, Madison, WI 53719). Conventional cytology and LBC cytology were performed at the Institute of Pathology (University of Freiburg, Germany), and residual LBC specimens were stored at room temperature for up to 3 years.

After staining with acetic acid and iodine, patients with visible acetic-white iodine-negative lesions were subjected to biopsy. At a subsequent visit, these patients were treated by the use of laser vaporization or conization (loop electrosurgical excision procedure [LEEP]) according to the cytological and histological results. Patients with abnormal Pap results and no visible acetic-white iodine-negative lesions were subjected to minicurettage and/or conization (LEEP). Patients who had a normal Pap test result and showed no visible lesion during colposcopy at the first visit or follow-up visits after treatment were not subjected to biopsy and were considered "histology normal."

HPV testing. The LBC specimens were tested under blinded conditions with HC2 (Qiagen Incorporated, Hilden, Germany) at the University of Heidelberg (Heidelberg, Germany), and with the AHPV assay at Gen-Probe Incorporated (San Diego, CA). The AHPV assay and the HC2 test are, respectively, nucleic acid amplification and signal amplification tests that allow the qualitative detection (positive/negative) of high-risk HPV genotypes from clinical specimens. The AHPV assay detects HPV E6/E7 mRNA from 14 high-risk HPVs (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) in clinical specimens (13). The AHPV assay process involves target capture with specific oligomers and magnetic microparticles, target mRNA amplification using transcription-mediated amplification, and signal detection using chemiluminescent probes. HC2 detects HPV DNA from 13 high-risk HPVs (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) (11). The HC2 test involves target capture using antibodies and chemiluminescent signal detection of high-risk HPV types based on signal amplification. The assays were performed according to the standard assay procedures described by the manufacturers. An analyte cutoff (CO) of 1.00 was used in the AHPV assay for determining HPV interpretation. Specimens processed with the HC2 test that yielded no pellet after centrifugation and were HC2 negative were excluded to avoid bias against HC2.

Specimens with discordant results between the AHPV assay and the HC2 test were genotyped using the Linear Array HPV genotyping test (Linear Array; Roche Diagnostics, Indianapolis, IN), which utilizes amplification by PCR and nucleic acid hybridization to allow genotyping of 37 high- and low-risk HPVs, including types 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39, and CP6108. Genotyping results were available for 48 of the 49 discordant samples (see Table 5).

TABLE 1. AHPV assay and HC2 test results compared to histology findings

Test	Status[a]	No. of specimens	No. (%) of positive specimens in AHPV	No. (%) of positive specimens in HC2
Histology	Normal	108	8 (7.4)	24 (22.2)
	CIN1	64	35 (54.7)	44 (68.8)
	CIN2	89	71 (79.8)	74 (83.1)
	CIN3	150	148 (98.7)	145 (96.7)
	Cervical carcinoma	13	12 (92.3)	11 (84.6)
Cytology	Normal	152	38 (25.0)	50 (32.9)
	ASC-US/ASC-H	13	11 (84.6)	9 (69.2)
	LSIL	44	22 (50.0)	34 (77.3)
	HSIL	212	200 (94.4)	201 (94.8)
	Cervical carcinoma	3	3 (100.0)	3 (100.0)
Total	All specimens	424	274 (65)	297 (70.0)

[a] ASC-H, ASC-US cannot exclude HSILs.

Statistical analysis. Detection rates for both the AHPV assay and the HC2 test were determined. Concordance between the two assays was determined. Sensitivity and specificity with 95% confidence intervals (CI) (based on McNemar's test) for the detection of cervical disease (CIN2+ and CIN3+) were determined for both assays and for cytology in comparison to histology results (considered the final diagnosis). McNemar's test was used to determine statistical significance ($P < 0.05$). No adjustments were made for multiple comparisons.

RESULTS

Prevalence of cervical disease in the population. A total of 424 specimens with histology, cytology, AHPV, and HC2 results were analyzed. Histology revealed that, of the 424 specimens, 108 (25.5%) were normal (25 of these had no visible lesion and did not have biopsy tissue taken), 64 (15.1%) were classified as CIN1, 89 (21%) as CIN2, 150 (35.4%) as CIN3, and 13 (3%) as cervical carcinoma (Table 1). Thus, the prevalence of cervical disease (CIN2+) in this population of women referred to colposcopy was 59.4%.

Detection of cervical disease by the AHPV assay, the HC2 test, and cytology in all women. The detection of high-risk HPV in cervical specimens by the AHPV assay and the HC2 test was analyzed in relation to histology and cytology status. Results are presented in Table 1. For both the AHPV assay and the HC2 test, the rate of positive results increased with the severity of cellular abnormality (from normal to high-grade squamous intraepithelial lesions [HSIL] or cervical carcinoma) as detected in cytology and histology. The AHPV assay detected 94.4% of HSIL and 100% of cervical cancers found by cytology. The AHPV assay exhibited lower reactivity than the HC2 test with specimens with normal histology (7.4% versus 22.2%, respectively; $P = 0.0003$) and CIN1 (54.7% versus 68.8%, respectively; $P = 0.0126$). The AHPV assay exhibited a reactivity similar to or higher than that of the HC2 test with specimens with biopsy-identified cervical disease (CIN2+): the AHPV assay and the HC2 test detected as positive 79.8% and 83.1% of CIN2, 98.7% and 96.7% of CIN3, and 92.3% and 84.6% of cervical carcinoma, respectively.

Sensitivity and specificity of assays for disease detection (CIN2+ and CIN3+). The sensitivity and specificity of the AHPV assay, the HC2 test, and cytology for the detection of

TABLE 2. Sensitivity and specificity of the AHPV assay, the HC2 test, and cytology for CIN2+ and CIN3+ specimens from all women independent of age and in women <30 and ≥30 years of age[a]

Status	Assay	Sensitivity		Specificity	
		%	95% CI	%	95% CI
All women					
CIN2+ (n = 252)	AHPV	91.70	(87.6–94.5)	75.00	(68.0–80.9)
	HC2	91.30	(87.1–94.2)	61.00	(53.6–68.0)
	Cytology	84.90	(80.0–88.9)	66.30	(58.9–72.9)
CIN3+ (n = 163)	AHPV	98.20	(94.7–99.4)	56.30	(50.3–62.2)
	HC2	95.70	(91.4–97.9)	46.00	(40.0–52.0)
	Cytology	93.90	(89.1–96.6)	54.40	(48.3–60.3)
Women <30 yr					
CIN2+ (n = 81)	AHPV	92.60	(84.8–96.6)	72.70	(59.8–82.8)
	HC2	92.60	(84.8–96.6)	50.90	(38.1–65.6)
	Cytology	85.20	(75.9–91.3)	58.20	(45.0–70.3)
CIN3+ (n = 41)	AHPV	97.60	(87.7–99.6)	47.40	(37.6–57.3)
	HC2	97.60	(87.7–99.7)	34.70	(25.9–44.7)
	Cytology	97.60	(87.7–99.6)	45.30	(35.6–55.3)
Women ≥30 yr					
CIN2+ (n = 171)	AHPV	91.20	(86.0–94.6)	76.10	(67.6–82.9)
	HC2	90.10	(84.7–93.7)	65.00	(56.0–73.0)
	Cytology	84.80	(78.7–89.4)	70.90	(62.2–78.4)
CIN3+ (n = 122)	AHPV	98.40	(94.2–99.6)	61.40	(53.9–68.5)
	HC2	95.10	(89.7–97.8)	52.40	(44.8–59.9)
	Cytology	92.60	(86.6–96.1)	60.20	(52.7–67.4)

[a] 95% confidence interval values calculated by the Score method are given.

CIN2+ and CIN3+ were calculated by comparison with histology results (probe excision or cone biopsies, considered the "gold standard") and are presented in Table 2.

The sensitivity of the AHPV assay for the detection of CIN2+ and CIN3+ specimens was 91.7% (95% CI: 87.6 to 94.5%) and 98.2% (94.7 to 99.4%), respectively. For the HC2 test, these values were 91.3% (87.1 to 94.2%) and 95.7% (91.1 to 97.9%), respectively. By comparison, the sensitivity of cytology was 84.9% (80.0 to 88.9%) for CIN2+ specimens and 93.9% (89.1 to 96.6%) for CIN3+ specimens. Thus, the AHPV assay had a sensitivity similar to that of the HC2 test for the detection of CIN2+ specimens, and both assays had a significantly higher sensitivity (6.4% and 6.8% higher, respectively) than cytology ($P = 0.0041$ for AHPV, and $P = 0.0094$ for HC2). For detection of CIN3+, the AHPV assay was significantly more sensitive than cytology (98.2% versus 93.9%; $P = 0.0348$) but not significantly more sensitive than HC2 (98.2% versus 95.7%; $P = 0.1573$).

The specificity of the AHPV assay for the detection of CIN2+ and CIN3+ specimens was 75.0% (95% CI: 68.0 to 80.9%) and 56.3% (50.3 to 62.2%), respectively. For the HC2 test, these values were 61.0% (53.6 to 68.0%) and 46.0% (40.0 to 52.0%), respectively. By comparison, the specificity of cytology was 66.3% (58.9 to 72.9%) for CIN2+ specimens and 54.4% (48.3 to 60.3%) for CIN3+ specimens. Thus, the AHPV assay had a significantly higher specificity than the HC2 test ($P < 0.0001$) and cytology ($P = 0.0163$) for the detection of CIN2+ specimens (Table 2). The difference in specificity for CIN2+ specimens was not statistically significant between cytology and the HC2 test ($P = 0.139$).

Detection of cervical disease by the AHPV assay, the HC2 test, and cytology in women by age group. The sensitivity and specificity of the AHPV assay, the HC2 test, and cytology for the detection of disease (CIN2+ and CIN3+) in women <30 versus ≥30 years of age are presented in Table 2. For detection of CIN2+, the AHPV assay had similar levels of sensitivity (92.6% versus 91.2%) and specificity (72.7% versus 76.1%) with specimens from both age groups. The HC2 test, on the other hand, showed comparable levels of sensitivity (92.6% versus 90.1%), but a lower specificity (50.9% versus 65.0%, respectively), with women <30 and women ≥30 years of age. For the detection of CIN3+, the AHPV assay had similar levels of sensitivity in all age groups, but a higher specificity with women ≥30 (61.4%) than with women <30 (47.4%). The HC2 test had a slightly lower sensitivity (95.1% versus 97.6%, respectively), but a higher specificity (52.4% versus 34.7%, respectively) with women ≥30 than with women <30.

Analysis of disease-positive specimens missed by the AHPV assay or the HC2 test. Disease-positive specimens that were missed by one of the HPV assays were genotyped using Linear Array; results are presented in Table 3. Four specimens could not be genotyped due to low sample volume. The AHPV assay missed 21 disease-positive specimens: 1 microinvasive squamous cell carcinoma (SCC), 2 CIN3, and 18 CIN2 specimens. The cervical carcinoma specimen that was missed by the AHPV assay contained HPV 53 (as determined by Linear Array) and was positive in HC2. Of the 18 CIN2 and 2 CIN3 specimens missed by the AHPV assay, 3 were not genotyped, 13 had either no HPV DNA or only low-risk DNA (5 of which were HC2 positive [$HC2^+$]), and 4 had high-risk HPV genotypes (2 of which were $HC2^+$) (Table 3).

The HC2 test missed 22 disease-positive specimens: 2 carcinomas, 5 CIN3, and 15 CIN2 specimens. Of the 2 cervical carcinoma specimens missed by the HC2 test, both were pos-

TABLE 3. Genotyping of disease-positive specimens missed by the AHPV assay or the HC2 test[a]

Disease-positive specimens missed by:	Histology	No. of samples	No. of samples not genotyped	Linear Array HPV genotyping (no. of samples)		
				Negative	Low-risk HPV	High-risk HPV
AHPV	CIN2	18 (7 $HC2^+$)	2 (1 $HC2^+$)	8 (1 $HC2^+$)	5 (4 $HC2^+$)	3 (1 $HC2^-$)
	CIN3	2 (1 $HC2^+$)	1 ($HC2^-$)	0	0	1 ($HC2^+$)
	Cervical carcinoma	1 ($HC2^+$)	0	0	1* ($HC2^+$)	0
HC2	CIN2	15 (4 $AHPV^+$)	1 ($AHPV^-$)	7 (7 $AHPV^-$)	1 ($AHPV^-$)	6 (4 $AHPV^+$)
	CIN3	5 (4 $AHPV^+$)	0	1 ($AHPV^-$)	0	4 (4 $AHPV^+$)
	Cervical carcinoma	2 (2 $AHPV^+$)	0	1 ($AHPV^+$)	0	1 ($AHPV^+$)

[a] When several HPV genotypes were detected in a specimen, the specimen was categorized according to the "worst" genotype present (high risk being worse than low risk, and low risk being worse than negative). Result of the other assay shown in parentheses. *, this specimen contained low-risk HPV-53 as the only HPV type.

itive by AHPV, 1 was negative for HPV as determined by Linear Array (but positive for HPV 16 in a Gen-Probe in-house genotyping assay), and the other contained HPV 18. Of the 15 CIN2 and 5 CIN3 specimens missed by the HC2 test, 1 was not genotyped, 9 had either no HPV DNA or only low-risk DNA (all of those were $AHPV^-$), and 10 had high-risk HPV genotypes (8 of which were $AHPV^+$).

Agreement between AHPV and HC2. The overall agreement between the AHPV assay and the HC2 test was 88.4%, the positive agreement was 87.9%, and the negative agreement was 89.8% (Table 4). The kappa coefficient for the overall agreement was 0.738, indicating good interrater agreement.

There were 49 discrepant specimens between the two assays, 48 of which were genotyped using Linear Array. Results are presented in Table 5. Thirty-six specimens were $AHPV^-$ and $HC2^+$, of which 35 were genotyped by Linear Array: three were negative for HPV DNA, 22 had low-risk HPV genotypes only, and 10 had a mixture of low-risk HPV genotypes and high-risk HPV genotypes present. Thirteen specimens were AHPV positive and HC2 negative and genotyped by Linear Array: one was HPV negative, and 12 had high-risk HPV genotypes.

Median RLU/CO values of the HC2 test in correlation to histology results. The median relative light unit (RLU)/CO values of the HC2 test and the AHPV assay for all 424 specimens are shown in Table 6. Interestingly, for both assays the RLU/CO values increase with the severity of the disease status but go down drastically from CIN3 to cervical cancer in the HC2 test in this aged (stored for up to 3 years) LBC specimen collection.

TABLE 4. Agreement between the AHPV assay and the HC2 test[a]

Test	Result	HC2		
		Positive	Negative	Total (%)
AHPV	Positive	261	13	274 (65)
	Negative	36	114	150 (36)
	Total (%)	297 (70)	127 (30)	424 (100)

[a] Values are number of specimens. Overall agreement: 88.4% (375/424). Positive agreement: 87.9% (261/424). Negative agreement: 89.8% (114/424). Kappa coefficient: 0.738 (95% CI, 0.67 to 0.81).

DISCUSSION

Analysis of the performance of the AHPV assay of clinical specimens from women referred to colposcopy demonstrated that the AHPV assay is able to detect HPV high-risk mRNA in LBC specimens with strong correlation to disease (sensitivity of 91.7% to detect CIN2+ and 98.2% to detect CIN3+). One limitation of this study is that specimens were stored for up to 3 years at room temperature before they were tested in the AHPV assay and the HC2 test. However, the values obtained for sensitivity and specificity in this study are in agreement with other referral studies showing an AHPV assay sensitivity of 91% (12) and 95% (27) for the detection of CIN2+ and 98% (12) and 97% (27) for the detection of CIN3+. The higher sensitivity of the AHPV assay for the detection of CIN3+ than of CIN2+ supports the observations of other authors that expression of HPV E6 and E7 mRNA (which is detected by the AHPV assay) increases with the increasing severity of cervical disease (2, 6, 8, 24).

It should be noted that patients in this cohort were referred to colposcopy due to a previously abnormal Pap test. There-

TABLE 5. Resolution of all AHPV assay and HC2 test discordant specimens[a]

Result	Histology	No. of samples	HPV type as determined by LA (no. of samples)****		
			Negative	Low risk	High risk
$AHPV^-/HC2^+$	Negative	16	1	9	6
	CIN1	11	1	8	2
	CIN2	7*	1	4	1
	CIN3	1***	1		
	Cervical Carcinoma	1		1**	
$AHPV^+/HC2^-$	Negative	1			1
	CIN1	2			2
	CIN2	4			4
	CIN3	4			4
	Cervical Carcinoma	2	1		1

[a] *, one could not be typed due to low sample volume; **, HPV 53; ***, microfocal CIN3 in conus; ****, high-risk determination is based on types detected in respective assays as high risk. HPV 66 was counted as high risk for the AHVP assay and as low risk for the HC2 test.

TABLE 6. Median RLU/CO values for the HC2 test and the AHPV assay in correlation to histology results

Histology result	Median RLU/CO	
	HC2	AHPV
Normal (n = 108)	0.40	0.00
CIN1 (n = 64)	3.89	8.75
CIN2 (n = 89)	32.28	11.01
CIN3 (n = 150)	53.36	11.37
Cervical carcinoma (n = 13)	4.90	11.42

fore, the sensitivity of the repeat cytology in this study is much higher than the reported sensitivity of 51% in routine screening studies (51%; 95% confidence interval [CI], 37% to 66%) (1).

Two studies mentioned above (12, 27) found a slightly (~1 to 4%) higher sensitivity for the HC2 test than for the AHPV assay. Similar to the results reported here, differences in sensitivity between the two assays in those two studies were also small and statistically not significant.

The AHPV assay missed 1 cervical carcinoma, 2 CIN3, and 18 CIN2 specimens. The cervical carcinoma specimen that was AHPV negative contained only HPV 53 by Linear Array. HPV 53 is found frequently in HPV-infected women but is very rarely associated with precancerous lesions or cervical cancer (19). The AHPV and HC2 assays are not designed to detect this genotype. The specimen was negative in the AHPV assay but positive in the HC2 test. It appears that the HC2 test was cross-reacting with HPV 53 in this specimen. Nonspecific detection of HPV 53 in the HC2 test has been described previously (4).

The AHPV assay missed only 4 cases of cervical disease resulting from specimens harboring high-risk HPV, if the Linear Array genotyping result is considered the gold standard for HPV DNA positivity. These misses (3 CIN2 and 1 microfocal CIN3) could be due to the fact that these specimens may not have expressed HPV E6/E7 mRNA despite the presence of a high-risk HPV DNA and persistent infection; indeed, it has been reported that not all HPV types detectable by a DNA test express HPV mRNA (6). Out of the 13 CIN2 specimens that were high-risk HPV DNA negative or only low-risk HPV DNA positive by Linear Array and were negative in the AHPV assay, 6 specimens were positive in the HC2 test. These specimens appear to be false positives in the HC2 test.

In summary, the AHPV assay actually missed 4 truly disease and high-risk HPV-positive specimens (3 CIN2 and 1 microfocal CIN3), while the HC2 test missed 11 (3 microfocal CIN2, 3 CIN2, 2 microfocal CIN3, 2 CIN3, and 1 cervical carcinoma); 9 of the specimens missed by the HC2 test were detected as positive by the AHPV assay.

Another limitation of this study is that biopsy specimens were not taken from patients that had normal colposcopy and cytology results. This might result in a lower disease prevalence in this cohort and a higher sensitivity for all three tests compared in this study.

The specificity of the AHPV assay for the detection of CIN2+ in this study is higher than that described in two other referral studies (42% [27] and 56% [12]), without a negative effect on sensitivity. One possible explanation is that specimens in this study were stored for a prolonged time at room temperature. This might lead to some degree of RNA degradation, affecting disease-negative specimens with low E6/E7 mRNA more than disease-positive specimens with abundant E6/E7 mRNA, thereby improving specificity without affecting sensitivity.

The specificity of the AHPV assay was 75.0% for the detection of CIN2+ and 56.3% for the detection of CIN3+. The lower specificity for CIN3+ versus CIN2+ detection is expected because of the fraction of CIN2 specimens that are disease positive and express E6/E7 mRNA. The significantly higher specificity of the AHPV assay than of the HC2 test in this and other studies can be explained by the fact that the AHPV assay exhibits lower reactivity in specimens with normal and CIN1 histology than the HC2 test (fewer false positives).

The AHPV assay specificity for CIN2+ detection (75.0%) was significantly higher than that for cytology (66.3%; P = 0.0163) and much higher than that for the HC2 test (61.0%; $P < 0.0001$). This is in agreement with other studies that have compared the performance of the AHPV assay versus the HC2 test (12, 14, 27). Szarewski and colleagues (27) also demonstrated that the AHPV assay is more specific than the Amplicor and Linear Array assays, two other HPV DNA amplification assays. Analysis of discordant results between the AHPV assay and the HC2 test by genotyping using Linear Array showed that the AHPV assay results agreed with genotyping results for the presence of high-risk HPV in 76% of specimens, while the HC2 test agreement with genotyping was only 22%. This finding can be attributed to the fact that the HC2 test has a much higher propensity to cross-react with some low-risk HPV genotypes (4, 11) than the AHPV assay (13).

Many studies tried to correlate the amount of viral DNA and E6/E7 mRNA with disease stage (3, 6, 8, 10, 15). However, results are conflicting because of the variation in sampling techniques and different methods used to calculate viral load. A couple of studies reported that the level of HPV E6/E7 mRNA is an independent prognostic factor in cervical cancer, whereas the HPV DNA copy number had no prognostic value (10, 15, 28). Some studies even found that DNA copy values went down in samples from cervical cancer patients (2, 5, 15, 30).

The median RLU/CO values of the HC2 test increased for our study population with the severity of the disease status but went down drastically in specimens with cervical cancer. Although the HC2 test is not designed to yield quantitative results, the RLU/CO values give a semiquantitative measure of high-risk HPV DNA load. HC2 test RLU/CO values have been used in other studies as a quantitative evaluation of viral load (9, 25, 26). Our findings suggest that the amount of HPV DNA is decreased in cervical cancer specimens, raising an interesting question regarding the importance of the cutoff for the HC2 test. If the cutoff of the HC2 test was raised from 1 to 10 as suggested by some authors (17), 37 out of 163 CIN3+ lesions (23%) would have been missed by the HC2 test in this patient cohort, including 7 out of 11 cervical cancer specimens. The median RLU/CO values in the AHPV assay were consistently high in disease-positive specimens (CIN2+), indicating that viral mRNA levels did not seem to decrease in cervical cancer specimens, as the viral DNA level seemed to do in some cervical cancer specimens. Since the AHPV assay is a qualitative

assay, an increase of mRNA levels, with severity of disease, would not be visible by looking at RLU/CO values.

Interestingly, one of the 2 cervical cancer specimens that were missed by the HC2 test was also negative for HPV DNA in the Linear Array assay, but positive for HPV mRNA in the AHPV assay.

After the current study was completed, several women returned for further follow-up visits after therapy. Among these women, three had been colposcopy/cytology and HC2 negative but positive in the AHPV assay. In all of these three patients, lesions (two CIN2 and one CIN1) were detected again a year later, and genotyping revealed the same HPV type (two HPV 16 and one HPV 18) that was detected before therapy. In another woman, an abnormal Pap test (HPV 33 positive) during pregnancy returned to normal 2 months after delivery. In this patient the AHPV test remained positive, while the HC2 test result was negative after delivery. In the same woman, an extensive CIN3 lesion (HPV 33) was detected 2 years later.

These anecdotal results from four follow-up patients that were colposcopy/cytology and HC2 negative but remained AHPV positive after treatment or spontaneous remission suggest that the AHPV assay is a very sensitive and specific tool for the follow-up testing of patients being treated for precancerous or cancerous lesions. Follow-up results of more women will need to be evaluated to confirm this observation.

The results from this referral study demonstrate that the Aptima HPV assay is able to detect HPV high-risk mRNA in retrospective clinical LBC specimens with high correlation to cervical disease. The AHPV assay was both significantly more sensitive and more specific than cytology. The AHPV assay had a sensitivity similar to that of the HC2 test and a significantly higher specificity for the detection of cervical disease (CIN2+) than the HC2 test.

In summary the AHPV assay had a sensitivity similar to that of the HC2 test but a significantly higher sensitivity than that of repeat cytology. The Aptima was significantly more specific for the detection of disease (CIN2+) than the HC2 test and repeat cytology. Therefore, we conclude from this study that the Aptima assay performs better than repeat cytology or the HC2 test in a referral population.

Due to its higher sensitivity and specificity, the AHPV assay might even be considered a primary screening test instead of cytology. A recently published study of a screening population reported that the AHPV assay had a higher sensitivity than cytology and a specificity similar to that of cytology, whereas the HC2 test had a higher sensitivity but lower specificity than cytology (18).

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