

A Study of Amplicor Human Papillomavirus DNA Detection in the Atypical Squamous Cells of Undetermined Significance–Low-Grade Squamous Intraepithelial Lesion Triage Study

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Abstract

We analyzed the performance of Amplicor for detecting carcinogenic human papillomavirus (HPV) infections and cervical precancer in women with an atypical squamous cells of undetermined significance (ASCUS) Pap and compared the results with Hybrid Capture 2 (hc2) in the ASCUS and low-grade squamous intraepithelial lesion (LSIL) triage study (ALTS). Baseline specimens collected from women referred into ALTS based on an ASCUS Pap result were prospectively tested by hc2 and retrospectively tested by Amplicor ($n = 3,277$). Following receiver-operator-characteristics curve analysis, Amplicor performance was analyzed at three cutoffs (0.2, 1.0, and 1.5). Paired Amplicor and hc2 results were compared for the detection of 2-year cumulative cervical intraepithelial neoplasia (CIN) grade 3 and more severe disease outcomes (CIN3+) and for the detection of 13 targeted carcinogenic HPV

types. Amplicor at the 0.2 cutoff had a higher sensitivity for the detection of CIN3+ (95.8% versus 92.6%, $P = 0.01$) but a much lower specificity (38.9% versus 50.6%, $P < 0.001$) than hc2. Amplicor at the 1.5 cutoff had an identical sensitivity for the detection of CIN3+ (92.6%) and a slightly lower specificity (47.5%; $P < 0.001$). The positive predictive value of hc2 was higher at all Amplicor cutoffs, whereas referral rates were significantly lower (53.2% for hc2 versus 64.1% at the 0.2 cutoff and 56.0% at the 1.5 cutoff, $P < 0.001$). Amplicor was more analytically specific for detecting targeted carcinogenic HPV types than hc2. Amplicor at the 1.5 cutoff had comparable performance with hc2. Whereas Amplicor missed more disease related to nontargeted types, hc2 was more likely to miss disease related to targeted types. (Cancer Epidemiol Biomarkers Prev 2009;18(5):1341–9)

Introduction

About 15 genotypes of the human papillomaviruses (HPV) are associated with the development of cervical cancer and its immediate precursor, cervical intraepithelial neoplasia grade 3 (CIN3) and carcinoma *in situ* (CIS). Although the vast majority of women are supposedly infected with HPV during their lifetime, only a small percentage of these women will develop a CIN3/CIS (1) and only a fraction of those become invasive cancer (2, 3).

Currently, screening for cervical cancer and its precursors is primarily based on cytology. A major limitation of cytologic screening is the low sensitivity of a single test, making repeated, regular screening at 1- to 2-year intervals necessary. Most nonnormal screening

results include low-grade squamous intraepithelial lesions (LSIL) and atypical squamous cells of undetermined significance (ASCUS; ref. 4). Although these abnormalities are typically cytomorphologic manifestations of innocuous, transient HPV infections, in 10% to 15% of women these cytologic changes are accompanied by an underlying histologic precancer (CIN3/CIS; refs. 5, 6). In fact, in absolute numbers, most precancers, including CIN2, are found at workup triggered by ASCUS and LSIL [versus high-grade squamous intraepithelial lesions (HSIL); ref. 7]. Therefore, LSIL and ASCUS cytology results require further diagnostic investigation to avoid missing high-grade disease.

Based on the central role of persistent, carcinogenic HPV in cervical carcinogenesis, carcinogenic HPV testing has recently been introduced into cervical cancer screening. Carcinogenic HPV testing has proven greater reproducibility (8, 9) and greater sensitivity for detection of cervical precancer (CIN3) and cancer (together, abbreviated here as \geq CIN3; refs. 10–14) than cytology. Recent American Society for Colposcopy and Cervical Pathology guidelines approve the use of carcinogenic

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HPV testing for triage of women with ASC-US, adjunct testing with cytology in primary screening for women age 30 years and older, postcolposcopy follow-up, and posttreatment monitoring (15). Currently, there is only one Food and Drug Administration–approved HPV detection assay, the Hybrid Capture 2 (hc2; Qiagen) test. hc2 uses RNA-DNA hybridization and signal amplification to detect the presence of any of the 13 carcinogenic types (HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, and HPV68) in aggregate; hc2 also cross-reacts with some untargeted, mostly noncarcinogenic HPV genotypes, especially HPV53, HPV67, HPV70, HPV82, and HPV66 (16), the latter of which has been recently reclassified as carcinogenic (17).

However, other tests are being developed and need to be evaluated for their clinical utility. One such test, the Amplicor HPV test (Roche Diagnostics), is a PCR-based assay that targets the same 13 carcinogenic genotypes as hc2. After amplification of a sequence in the HPV *L1* gene, labeled probes are hybridized to the amplified DNA and the absorbance is measured. Despite the use of Amplicor in several studies (18-23), there has been no formal, independent evaluation of Amplicor cutoffs for detecting the presence of a carcinogenic genotype (analytical sensitivity) or the presence or risk of cervical precancer (clinical sensitivity).

The aim of this study was to formally evaluate cutoff levels for Amplicor positivity and to analyze the analytic and clinical performance of Amplicor in detecting carcinogenic HPV and related disease within the ASCUS-LSIL triage study (ALTS).

Subjects and Methods

Study Design and Population. ALTS was a multicenter, randomized trial conducted by the National Cancer Institute (NIH, Rockville, MD) comparing three management strategies for women with ASCUS ($n = 3,488$) or LSIL ($n = 1,572$) cytology (referral cytology before revision of the Bethesda terminology). Women were either managed with immediate colposcopy (IC arm, referral regardless of test results), HPV triage (HPV arm, referral to colposcopy if enrollment hc2 result was positive or missing, or, for patients' safety, if the enrollment cytology result was HSIL), or by conservative management (referral to colposcopy only if enrollment cytology result was HSIL). During the 2-y follow-up, women in the three arms of the study were reevaluated by cytology every 6 mo and sent to colposcopy if cytology was called HSIL by the clinical center pathologists.

At all visits, women received a pelvic exam and two cervical specimens were collected, one into PreservCyt (Cytoc) for cytology and hc2 testing and a second cervical specimen into specimen transport medium (STM; Qiagen). 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.

HPV DNA Testing. During ALTS, residual PreservCyt specimens were tested by hc2 according to the manufacturer's instructions, defining hc2 signal strength by relative light units/positive control.

Amplicor testing was done on archived aliquots of the enrollment STM specimens according to manufacturer's specifications for women referred into ALTS for an ASCUS Pap smear. One hundred microliters STM aliquots were subjected to automated sample preparation for DNA extraction of up to 96 specimens at a time on the Qiagen MDx platform (using the MinElute medium MDx kit and the manufacturer's instructions).

HPV genotyping data were obtained using two methods, the Line Blot Assay (LBA) and the commercialized version of LBA, Linear Array (LA). Both are L1 consensus primer–based PCR assays using PGM09/11 primers. LBA was done on the STM specimens as previously described (24-26) for 27 or 38 HPV genotypes, including the 13 carcinogenic HPV genotypes targeted by hc2 and Amplicor as well as the untargeted HPV genotypes with which hc2 is most likely to cross-react. Aliquots of the archived enrollment STM specimens from women referred into ALTS because of ASCUS were retested using LA, which tests for 37 of the 38 HPV genotypes detected by LBA, excluding HPV57, as previously described (27). LA was used according to the manufacturer's instructions in the product insert, except that an automated sample preparation for DNA extraction was used as described above for Amplicor.

Pathology and Treatment. Clinical management was based on the clinical center pathologists' (CC pathology) cytologic interpretations and histologic diagnoses as previously described (5, 6, 28, 29). Referral smears, ThinPreps, and histology slides were also sent to the Pathology Quality Control Group (QC Pathology) based at the Johns Hopkins Hospital for review, including computer-assisted review, and secondary diagnoses as previously described (5, 6, 28, 29). Women with a CC diagnosis of CIN2 or more severe (CIN2+) or a QC diagnosis of CIN3 or more severe (CIN3+) were offered excisional treatment by loop electrosurgical excision procedure. In addition, all women with persistent mild abnormalities (e.g., LSIL or carcinogenic HPV-positive ASCUS) at the time of exit from the study were also offered loop electrosurgical excision procedure.

Statistical Analysis. Of the 3,488 women referred into ALTS because of an ASCUS Pap, 3,442 (98.7%) had Amplicor results, 3,326 (95.4%) had hc2 results, and 3,277 (94.0%) had both hc2 and Amplicor results, constituting the analysis population. There were no differences in the frequencies by study arm, colposcopy at enrollment, QC referral cytology, QC enrollment cytology, and HPV risk groups between the women included in the analysis ($n = 3,277$) and those excluded due to missing test results ($n = 211$). There were minor differences of enrollment at study centers (Oklahoma: 7.6% versus 14.2%, respectively, and Pennsylvania: 25.6% and 19.3%, respectively, $P = 0.02$) between women included and excluded from the study. Among the women included in this analysis, 3,167 of 3,277 (96.6%) had LBA results, 3,277 of 3,277 (100%) had LA results, and 3,167 of 3,277 (96.6%) had LBA or LA results for detection of individual HPV genotypes.

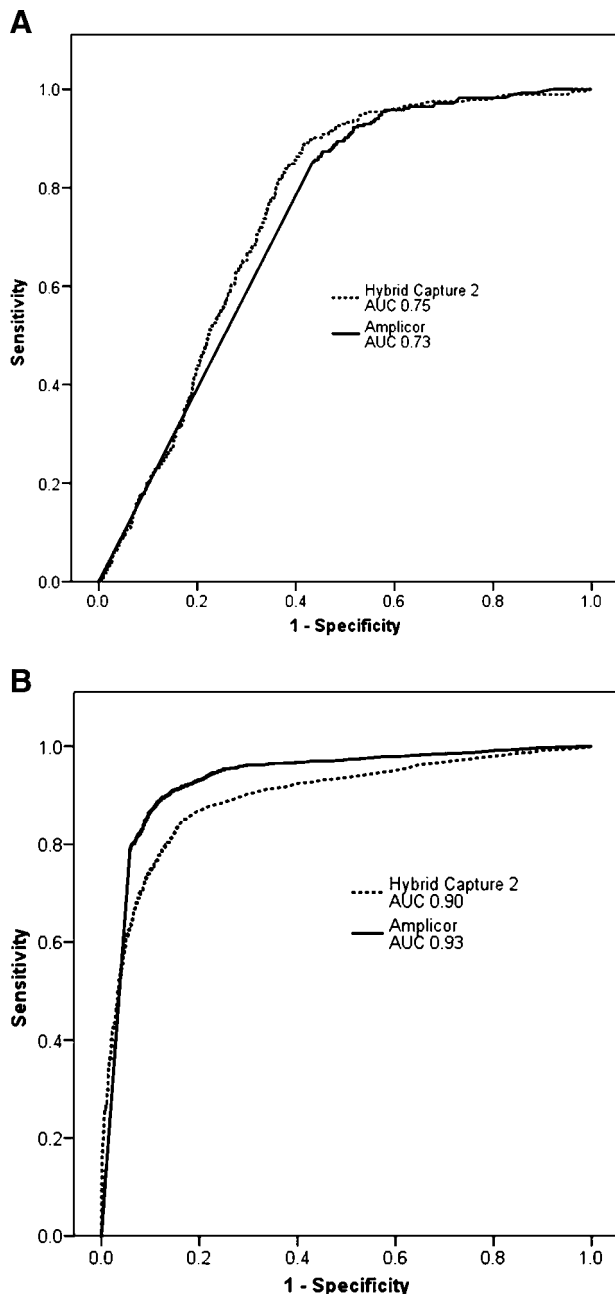


Figure 1. Clinical and analytical performance of Amplicor and hc2 analyzed in ROC curve analysis with areas under the curve (AUC) calculation to detect 2-y cumulative CIN3 or more severe (CIN3+) cases as diagnosed by quality control pathology (A) and 13 targeted carcinogenic HPV genotypes as detected by defined by LA and/or LBA (B).

Unlike hc2, Amplicor has an internal control based on the amplification of the β -globin gene. Because Amplicor sample adequacy is defined by both the β -globin and the HPV absorbance, sample adequacy was affected when both cutoffs were altered. In all women referred for ASCUS cytology that were tested for Amplicor, 3,421 of

3,442 (99.4%) specimens had a β -globin cutoff ≥ 0.2 and 3,277 of 3,442 (95.2%) specimens had a β -globin cutoff ≥ 1.0 . To analyze whether the different β -globin cutoff levels influenced the clinical performance, we compared receiver-operator-characteristic (ROC) curves based on HPV cutoffs for the detection of QC CIN3+ in the complete study at both β -globin cutoffs. Both curves had identical shapes, indicating that there was no difference in performance characteristics between a 0.2 and 1.0 β -globin cutoff (data not shown). In agreement with previous studies, we used a 0.2 cutoff for β -globin positivity in the subsequent analyses.

ROC curve analysis was done for Amplicor by calculating the sensitivity and 1-specificity for detecting 2-year cumulative CIN3+ as diagnosed by QC Pathology in all study arms at all cutpoints. Youden's index (YI) was calculated as sensitivity + specificity - 1. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and YI were calculated using Amplicor cutpoints of 0.2, 1.0, and 1.5 for the following end points: QC Pathology-diagnosed 2-year cumulative CIN3+, CC Pathology-diagnosed 2-year cumulative CIN2+, QC Pathology-diagnosed CIN3+ at enrollment among women in the IC arm, and CC Pathology-diagnosed CIN2+ at enrollment among women in the IC arm. The Amplicor results were compared between different cutpoints and to hc2 results as the reference standard. Differences in test positivity (referral rate), test sensitivity, and specificity for CIN3+ and CIN2+ were tested for statistical significance using an exact McNemar's χ^2 test. Differences in PPV and NPV were tested for statistical significance according to the method developed by Leisenring and Pepe (30).

Detection of carcinogenic HPV by LA and hc2 was compared by calculating κ values and percent total agreement. Differences in detection were tested for statistical significance using an exact McNemar's χ^2 test. Paired results were stratified by enrollment ThinPrep cytology results (ASC-US, LSIL, ASC-H, HSIL, according to the revised Bethesda terminology) as rendered by CC pathology. They were also stratified using an a priori established HPV categorization according to cervical cancer risk based on the detection of these types by LBA or LA: 1, positive for HPV16; 2, else positive for HPV18; 3, else positive for any carcinogenic HPV genotype and negative for HPV16 and HPV18 (HR11); 4, else positive for any noncarcinogenic HPV genotypes and negative for all carcinogenic genotypes; or 5, PCR negative (HPV16 > HPV18 > carcinogenic HPV exc. HPV16 and HPV18 > noncarcinogenic HPV > PCR negative). In the case of multitype infections, the result was assigned to the highest risk category.

We also compared the percent positive tests for hc2 and Amplicor at the 0.2 and 1.5 cutpoint for a second hierarchical classification of HPV risk groups [HPV16 > HPV18, other carcinogenic types (HR11) > HPV66 > HPV53/67/70/82 > other HPV], which included additional categories for untargeted HPV genotypes commonly detected by hc2, to compare and contrast the analytic specificity of the two assays. Significance testing was based on Pearson's χ^2 test. All statistical tests were two-sided and considered to be significant at $P < 0.05$. Bonferroni's correction was used to adjust for multiple statistical testing in comparing the clinical performance

Table 1. Clinical performance of carcinogenic HPV detection by Amplicor and hc2 for 2-year cumulative CIN3 or more severe (CIN3+) as diagnosed by quality control pathology and 2-year cumulative CIN2+ diagnosed by clinical center pathology (n = 3,286) as well as restricted to the IC arm (n = 1,081) in women referred into ALTS for an ASCUS Pap

Test	Sens, % (CI)	P	Spec, % (CI)	P	PPV, % (CI)	P	NPV, % (CI)	P	Youden	Referral (%)	P	Invalid	n
All study arms, outcome = QC–CIN3+ (n = 285)													
Amplicor 0.2	95.8 (92.8-97.8)	0.01	38.9 (37.1-40.6)	<0.001	13.0 (11.6-14.5)	<0.001	98.98 (98.22-99.47)	0.13	34.7	64.1	<0.001	9	3,277
Amplicor 1.0	93.3 (89.8-95.9)	0.80	44.6 (42.8-46.4)	<0.001	13.8 (12.3-15.4)	<0.001	98.59 (97.81-99.15)	0.89	37.9	58.7	<0.001	9	3,277
Amplicor 1.5	92.6 (89.0-95.4)	1.00	47.5 (45.7-49.3)	<0.001	14.4 (12.8-16.1)	0.007	98.54 (97.78-99.10)	0.74	40.2	56.0	<0.001	9	3,277
hc2	92.6 (89.0-95.4)	Reference	50.6 (48.8-52.4)	Reference	15.1 (13.5-16.9)	Reference	98.64 (97.92-99.15)	Reference	43.2	53.2	Reference		3,286
All study arms, outcome = CC–CIN2+ (n = 354)													
Amplicor 0.2	93.0 (90.4-95.1)	0.12	41.1 (39.2-42.9)	<0.001	22.1 (20.4-24.0)	<0.001	97.02 (95.88-97.92)	0.77	34.05	64.1	<0.001	9	3,277
Amplicor 1.0	90.6 (87.7-93.0)	0.74	47.0 (45.1-48.9)	<0.001	23.5 (21.7-25.5)	<0.001	96.52 (95.40-97.43)	0.15	37.59	58.7	<0.001	9	3,277
Amplicor 1.5	89.4 (86.4-92.0)	0.20	50.1 (48.2-51.9)	<0.001	24.4 (22.4-26.4)	<0.001	96.33 (95.22-97.24)	0.05	39.45	56.0	<0.001	9	3,277
hc2	91.0 (88.2-93.4)	Reference	53.7 (51.8-55.5)	Reference	26.2 (24.1-28.3)	Reference	97.08 (96.11-97.86)	Reference	44.7	53.2	Reference		3,286
Immediate colposcopy arm, outcome = QC–CIN3+ (n = 55)													
Amplicor 0.2	92.7 (82.4-98.0)	0.06	36.2 (33.3-39.3)	<0.001	7.2 (5.4-9.4)	0.0006	98.93 (97.29-99.71)	0.24	29.0	65.2	<0.001	2	1,079
Amplicor 1.0	92.7 (82.4-98.0)	0.38	41.6 (38.6-44.7)	<0.001	7.9 (5.9-10.2)	0.049*	99.07 (97.64-99.75)	0.48	34.3	60.1	<0.001	2	1,079
Amplicor 1.5	90.9 (80.1-97.0)	0.63	44.6 (41.6-47.7)	0.038*	8.1 (6.1-10.5)	0.35	98.92 (97.49-99.65)	0.53	35.5	57.2	0.03*	2	1,079
hc2	90.9 (80.1-97.0)	Reference	48.0 (44.9-51.1)	Reference	8.6 (6.4-11.1)	Reference	98.99 (97.67-99.67)	Reference	38.9	54.0	Reference		1,081
Immediate colposcopy arm, outcome = CC–CIN2+ (n = 113)													
Amplicor 0.2	91.7 (84.9-96.2)	0.23	37.7 (34.7-40.9)	<0.001	14.2 (11.7-17.0)	<0.001	97.60 (95.49-98.90)	0.91	29.5	65.2	<0.001	2	1,079
Amplicor 1.0	89.0 (81.6-94.2)	1.00	43.1 (40.0-46.3)	<0.001	15.0 (12.3-17.9)	0.0010	97.21 (95.18-98.55)	0.57	32.1	60.1	<0.001	2	1,079
Amplicor 1.5	88.1 (80.5-93.5)	1.00	46.3 (43.1-49.5)	0.019*	15.6 (12.8-18.7)	0.042*	97.19 (95.24-98.49)	0.50	34.4	57.2	0.03*	2	1,079
hc2	89.9 (82.7-94.9)	Reference	50.0 (46.8-53.2)	Reference	16.8 (13.8-20.1)	Reference	97.79 (96.07-98.89)	Reference	39.9	54.0	Reference		1,081

NOTE: "Invalid" indicates samples with a globin result below 0.2 and an HPV result below the respective cutoff. Differences in sensitivity, specificity, and referral were tested for statistical significance using an exact McNemar's χ^2 test. Differences in PPV and NPV were determined by a method developed by Leisenring and Pepe (30).

Abbreviations: Sens, sensitivity; Spec, specificity; CI, 95% confidence interval.

*Not significant after correction for multiple testing.

and the HPV detection at different Amplicor cutoffs. Statistical analyses were done using SAS, version 9.1 (SAS Institute).

Results

Analysis of Amplicor Cutoffs. We analyzed the HPV cutoffs and computed YI for every cutpoint analyzed to identify optimal tradeoffs between sensitivity and specificity for CIN3+ detection at a minimum sensitivity of 90% or greater. In that region, ROC analysis showed a flat curve and did not reveal a clear cutoff value (Fig. 1A). In agreement with those data, the YI versus cutpoint plot did not show any distinguishing peaks for YI, except for a slight elevation at the 1.5 cutpoint (YI = 0.4; not shown). Previous studies have used the 0.2 cutoff, which showed one of the lowest YIs in our analysis (YI = 0.35). Based on this analysis and the previously published literature, we decided to evaluate three absorbance cutpoints (0.2, 1.0, and 1.5) for their analytical and clinical performance of Amplicor in ALTS. Similarly, we analyzed Amplicor HPV cutoffs to detect the 13 targeted carcinogenic types as detected by LBA or LA. The ROC curve showed a greater area under the curve for carcinogenic HPV than for CIN3+ detection, suggesting that Amplicor better discriminates between carcinogenic HPV DNA positive versus negative than between 2-year cumulative CIN3+ versus <CIN3+ (Fig. 1B).

Clinical Performance of Amplicor HPV Detection.

Using QC Pathology–diagnosed 2-year cumulative CIN3+ as our primary end point, we compared the clinical performance of Amplicor at three cutpoints (0.2, 1.0, and 1.5) and hc2 (Table 1). As the cutoff value for Amplicor was changed from 0.2 to 1.0 to 1.5, sensitivity changed from 95.8% to 93.3% to 92.6%, respectively. Accordingly, specificity changed from 38.9% to 44.6% to 47.5% at the three cutoffs, respectively. By comparison, hc2 had a sensitivity of 92.6% and a specificity of 50.6%. The difference in sensitivity between Amplicor and hc2 was only significant at the 0.2 cutoff, whereas Amplicor was less specific than hc2 at all cutpoints.

The PPV of Amplicor was 13.0% at the 0.2 cutpoint, 13.8% at the 1.0 cutpoint, and 14.4% at the 1.5 cutpoint. All were significantly less than the 15.1% PPV for hc2. The NPV of Amplicor was 99.98% at the 0.2 cutpoint, 98.59% at the 1.0 cutpoint, and 98.54% at the 1.5 cutpoint. These NPVs were not significantly different from a NPV of 98.64% for hc2.

At the 0.2 cutpoint, Amplicor had a referral rate of 64.1% that was reduced to 58.7% and 56.0% at the 1.0 and 1.5 cutpoints, respectively, but all referral rates were still significantly higher than the referral rate of 53.2% for hc2 ($P < 0.001$ for all cutpoints).

We extended our analysis to the clinical center diagnosis of CIN2+ as a less strict outcome and restricted it to the immediate colposcopy arm. These modified end points yielded very similar results. Most importantly, neither assay showed qualitative differences at the

Table 2. Comparison of and agreement between Amplicor and hc2 for the detection of carcinogenic HPV for all paired tests stratified by the enrollment cytology result and HPV risk group as detected by LA and/or LBA for women referred into ALTS for an ASCUS Pap at two Amplicor cutoffs (0.2 and 1.5)

Group	n	hc2+ (%)	Amp+ (%)	hc2+/AMP+ (%)	hc2+/AMP- (%)	hc2-/AMP+ (%)	hc2-/AMP- (%)	κ	Agreement (%)	P
AMP 0.2/hc2, cytology stratification										
All	3,277	1,742 (53.16)	2,102 (64.14)	1,561 (47.64)	181 (5.52)	541 (16.51)	994 (30.33)	0.55	77.97	<0.001
ASC-US	819	455 (55.56)	534 (65.20)	408 (49.82)	47 (5.74)	126 (15.38)	238 (29.06)	0.56	78.88	<0.001
LSIL	576	530 (92.01)	501 (86.98)	485 (84.20)	45 (7.81)	16 (2.78)	30 (5.21)	0.44	89.41	<0.001
ASC-H	128	104 (81.25)	107 (83.59)	97 (75.78)	7 (5.47)	10 (7.81)	14 (10.94)	0.54	86.72	0.47
HSIL	176	171 (97.16)	172 (97.73)	169 (96.02)	2 (1.14)	3 (1.70)	2 (1.14)	0.43	97.16	0.65
AMP 0.2/hc2, HPV stratification										
HPV16	577	501 (86.83)	549 (95.15)	496 (85.96)	5 (0.87)	53 (9.19)	23 (3.99)	0.40	89.95	<0.001
HPV18	160	124 (77.50)	152 (95.00)	121 (75.63)	3 (1.88)	31 (19.38)	5 (3.13)	0.16	78.75	<0.001
HR11	1,056	842 (79.73)	997 (94.41)	831 (78.69)	11 (1.04)	166 (15.72)	48 (4.55)	0.29	83.24	<0.001
Non-HR	476	151 (31.72)	120 (25.21)	40 (8.40)	111 (23.32)	80 (16.81)	245 (51.47)	0.02	59.87	0.025
Neg	898	51 (5.68)	206 (22.94)	9 (1.00)	42 (4.68)	197 (21.94)	650 (72.38)	-0.02	73.39	<0.001
AMP 1.5/hc2, cytology stratification										
All	3,277	1,742 (53.16)	1,834 (55.97)	1,470 (44.86)	272 (8.30)	364 (11.11)	1,171 (35.73)	0.61	80.59	<0.001
ASC-US	819	455 (55.56)	464 (56.65)	383 (46.76)	72 (8.79)	81 (9.89)	283 (34.55)	0.62	81.32	0.47
LSIL	576	530 (92.01)	466 (80.90)	454 (78.82)	76 (13.19)	12 (2.08)	34 (5.90)	0.36	84.72	<0.001
ASC-H	128	104 (81.25)	102 (79.69)	95 (74.22)	9 (7.03)	7 (5.47)	17 (13.28)	0.60	87.50	0.62
HSIL	176	171 (97.16)	166 (94.32)	164 (93.18)	7 (3.98)	2 (1.14)	3 (1.70)	0.38	94.89	0.10
AMP 1.5/hc2, HPV stratification										
HPV16	577	501 (86.83)	530 (91.85)	483 (83.71)	18 (3.12)	47 (8.15)	29 (5.03)	0.41	88.73	<0.001
HPV18	160	124 (77.50)	148 (92.50)	119 (74.38)	5 (3.13)	29 (18.13)	7 (4.38)	0.20	78.75	<0.001
HR11	1,056	842 (79.73)	917 (86.84)	790 (74.81)	52 (4.92)	127 (12.03)	87 (8.24)	0.40	83.05	<0.001
Non-HR	476	151 (31.72)	58 (12.18)	13 (2.73)	138 (28.99)	45 (9.45)	280 (58.82)	-0.06	61.55	<0.001
Neg	898	51 (5.68)	112 (12.47)	4 (0.45)	47 (5.23)	108 (12.03)	739 (82.29)	-0.03	82.74	<0.001

NOTE: HPV risk groups are determined by the presence of the respective type in LA or LBA. Differences were tested for statistical significance using an exact McNemar's χ^2 test. Abbreviations: AMP, Amplicor; HR11, all targeted carcinogenic types without HPV16 and HPV18; Non-HR, all HPV types except targeted carcinogenic types; Neg, negative for HPV by LA or LBA.

Table 3. Detection of cases positive for single HPV genotypes ($n = 815$) by Amplicor and hc2 stratified by HPV risk groups (defined by LA and/or LBA test results) in women referred to ALTS for an ASCUS Pap

Type	hc2	Amplicor at 0.2 cutpoint	<i>P</i>	Amplicor at 1.5 cutpoint	<i>P</i>
16	75/102 (73.5%)	99/107 (92.5%)	<0.001	93/107 (86.9%)	<0.001
18	22/35 (62.9%)	32/35 (91.4%)	0.001	32/35 (91.4%)	0.001
HR11	216/307 (70.4%)	294/320 (91.9%)	<0.001	266/320 (83.1%)	<0.001
66	16/26 (61.5%)	5/27 (18.5%)	0.001	0/27 (0%)	<0.001
53/67/70/82	39/72 (54.2%)	18/76 (23.7%)	0.009	5/76 (6.6%)	<0.001
Other	26/237 (11.0%)	60/249 (24.1%)	<0.001	33/249 (13.3%)	0.04*

NOTE: Cases were attributed to HPV risk groups hierarchically as follows: HPV16 > HPV18, other carcinogenic types (HR11) > HPV66 > HPV53/67/70/82 > other HPV. Single HPV genotype infections were determined by LA or LBA positivity. Differences were tested for statistical significance using an exact McNemar's χ^2 test.

*Not significant after correction for multiple testing.

different end points; all performance measures changed in the same direction and order of magnitude. YI, a measure of test accuracy, was higher at all hc2 cutoffs compared with Amplicor.

Agreement between hc2 and Amplicor. At the 0.2 cutoff, 2,102 of 3,277 (64.1%) specimens tested positive by Amplicor, whereas 1,742 (53.2%) tested positive by hc2 ($P < 0.001$). At the higher 1.5 cutoff, 1,834 (56.0%) specimens tested positive by Amplicor, which was still greater than hc2 ($P < 0.001$). The overall agreement between hc2 and Amplicor was 78.0% ($\kappa = 0.55$) at the 0.2 Amplicor cutoff and 80.6% ($\kappa = 0.61$) at the 1.5 Amplicor cutoff.

At all cutoffs, Amplicor had a higher sensitivity to detect infections by HPV16, HPV18, and all other targeted carcinogenic types combined ($P < 0.001$), whereas nontargeted noncarcinogenic types were more frequently detected by hc2 than Amplicor at the 0.2 cutoff ($P = 0.03$) and at the 1.5 cutoff ($P < 0.001$; Table 2). Among women with LSIL cytology, hc2 was more likely to test positive than Amplicor at either the 0.2 or the 1.5 cutoff ($P < 0.001$). Stratification of the LSIL group by HPV risk groups showed that the discordance between hc2 and Amplicor was the result of more noncarcinogenic HPV-positive infections testing positive by hc2 than Amplicor (data not shown). Overall, a very high agreement between Amplicor and hc2 was observed for the HSIL cytology group at both Amplicor cutoffs (97.2% at the 0.2 cutoff and 94.9% at the 1.5 cutoff, respectively).

Analytical Performance of Amplicor. To analyze the analytical performance of Amplicor, the Amplicor results at the 0.2 and 1.5 cutoffs were compared with the HPV genotyping results from all samples with single HPV genotype infections ($n = 815$). Single-type infections were grouped in six HPV risk categories: HPV16, HPV18, other targeted carcinogenic types (HR11), HPV66 (as a nontargeted, but carcinogenic type that is frequently detected by hc2), HPV53/67/70/82 (as noncarcinogenic types that are frequently detected by hc2), and other HPV types. At both cutoffs, Amplicor showed significantly higher detection rates of targeted types than hc2 (Table 3). In contrast, Amplicor had significantly lower detection rates for nontargeted types that are frequently detected by hc2. Only the detection rate of other noncarcinogenic types (excluding HPV53, HPV67, HPV70, and HPV82) was slightly greater for Amplicor than for hc2 (significant only at the 0.2 cutoff), and

specifically HPV61 was more frequently detected by Amplicor than hc2 ($P = 0.008$ at the 1.5 cutoff; Supplementary Table S1). When increasing the cutoff from 0.2 to 1.5 for Amplicor, a smaller fraction of positive results for targeted HPV genotypes at the 0.2 cutoff were reclassified as negative at the 1.5 cutoff (34 of 462; 7.4%) than was observed for the nontargeted HPV genotypes [45 of 352 (12.8%); $P = 0.01$], which explains the increased clinical accuracy at the higher cutoff point compared with the lower cutoff point.

CIN3+ Cases Not Detected by Amplicor and hc2. We analyzed the HPV genotypes found in the CIN3+ cases missed by either Amplicor and/or hc2. Twelve and 21 cases of CIN3+ were negative by Amplicor at the 0.2 and 1.5 cutoffs, respectively, whereas 21 cases tested negative by hc2. Cases missed by Amplicor at either cutoff point primarily tested positive for nontargeted HPV genotypes, whereas cases missed by hc2 primarily tested positive for targeted HPV types (Table 4). In addition, we analyzed the HPV genotypes at the time of CIN3+ diagnosis based on the LBA results (LA data were not available for the later time points). Despite some genotype changes that suggest incident infections might have caused these cases, still hc2 missed more cases related to HPV16, whereas Amplicor was more likely to miss cases related to nontargeted types (Supplementary Table S2).

Discussion

With HPV detection most likely becoming an integral component of primary cervical cancer screening soon, it is important to identify the best assays to be used in screening. For several years, hc2 has been the only Food and Drug Administration–approved HPV detection assay and it has been used in the majority of cervical cancer screening studies. Based on a wealth of studies using hc2, there is excellent knowledge about the strengths and weaknesses of this test. Cross-reactivity with a number of nontargeted types has been reported for hc2 (16); the sensitivity of the assay can be compromised in less than optimal settings (31); and there is no specimen adequacy control included in this assay. Despite these limitations, hc2 has been proven a very robust assay with good clinical performance. Still, with HPV testing at the doorstep to screening programs around the world, it is necessary to have more than one

reliable HPV test and performance data on new assays are urgently required.

Based on the design of the Amplicor HPV assay, it might be used for similar applications as hc2: The test targets the same 13 carcinogenic HPV types and gives a dichotomous result for the presence of any of these types. One theoretical advantage of Amplicor compared with hc2 is that it uses amplification of the β -globin gene as an internal specimen adequacy control. The analysis of the globin results in our study showed that almost all samples contained DNA adequate for amplifying β -globin; only 21 samples (0.6%) had a β -globin cutoff below 0.2. Only nine samples were found to be invalid based on β -globin and HPV absorbance values; consequently, our results would not have changed if the adequacy control was disregarded completely. The very high percentage of adequate samples in our study might be related to the rigorous sampling within a clinical trial. In regular clinical practice with less rigorous collection procedures, a specimen adequacy control might be more important.

The ALTS trial offers a unique opportunity to analyze the performance of HPV detection assays because of the excellent disease ascertainment: 2-year follow-up to overcome limitations in sensitivity in colposcopy, colposcopic evaluation of women who returned for their exit visit, and rigorous review of histologic end points. Meanwhile, HPV genotyping data obtained with several HPV typing assays are available for ALTS samples for reference and to examine the analytic specificity of each pooled probe assay (27).

However, we acknowledge that the results from ALTS primarily relate to the triage of equivocal cytology and cannot be translated directly to primary screening applications. The HPV type distribution of HPV-positive cytologically normal specimens is different than that of ASCUS/LSIL cytology and than that of HSIL cytology (32). Also, with a median age of 25 years, the ALTS population was very young. A limitation of our study was the use of archival STM specimens rather than PreservCyt in the Amplicor assay, for which the Amplicor assay protocol had not been developed. Yet, its overall performance for detection of CIN3+ and its

comparability with hc2 shows that this was of a minor concern.

We performed a thorough evaluation of analytical and clinical sensitivity of Amplicor at different cutoffs. Although the 0.2 cutoff has been widely used in previous clinical studies (18-23), there are no published data on a formal evaluation of Amplicor performance at different cutoff levels. We observed a very low dynamic range of Amplicor absorbance values, with most samples showing a very high or completely saturated result; only 8% of the samples had absorbance values between 0.2 and 1.5. This contrasts with the wide dynamic range found for hc2 results that has been shown to correlate with viral load in some studies (33, 34).

In our analysis, we found that the Amplicor 1.5 cutoff was only slightly less sensitive but considerably more specific than the 0.2 cutoff. In agreement with that, YI was highest at the 1.5 cutoff. At the 1.5 cutoff, the clinical performance of Amplicor most closely resembled that of hc2, although its specificity (47.5% versus 50.6%, $P < 0.001$) and PPV (14.4% versus 15.1%, $P = 0.007$) was still slightly lower than hc2.

At the 0.2 cutoff, Amplicor had 11% higher positivity rates than hc2, which would result in a significantly higher referral of women with ASCUS cytology to colposcopy (64% versus 53%, $P < 0.001$). It remains to be determined whether the HPV-based triage would still be cost-effective at these increased referral rates (35). Although our data support using the 1.5 Amplicor cutoff, deciding which cutoff to use certainly depends on the application and the relative importance of sensitivity and specificity for that application. If used as a screening test with the goal to achieve maximum sensitivity and NPV to increase the screening intervals, making HPV testing cost-effective (36), choosing a lower cutoff may be appropriate. However, using a low cutoff in primary screening must await an appropriate triage test to be viable to avoid unnecessary high colposcopy referral rates.

We thoroughly studied the cross-reactivity pattern of Amplicor by evaluating the Amplicor levels in cases with single genotype infections by combining two HPV genotyping results to maximize HPV genotype sensitivity. At the 0.2 cutoff, there was a general low-level

Table 4. HPV genotypes (defined by LA and/or LBA test results) found in 2-year cumulative CIN3 or more severe (CIN3+) cases as diagnosed by QC pathology missed by Amplicor and hc2 in women referred into ALTS for an ASCUS Pap

Type	Total missed	Missed by Amplicor at 0.2 cutpoint	Missed by Amplicor at 1.5 cutpoint	Missed by hc2	Missed by Amplicor at 0.2 cutpoint and hc2	Missed by Amplicor at 1.5 cutpoint and hc2
16	4	0	2	2	0	0
18	1	0	0	1	0	0
HR11	8	2	4	6	1	2
66	1	1	1	0	0	0
53/67/70/82	5	2	5	2	2	2
Other	5	3	4	4	3	4
NA	7	4	5	6	3	4
Total	31	12	21	21	9	12

NOTE: Cases were attributed to HPV risk groups in hierarchical order: HPV16 > HPV18 > HR11 (targeted carcinogenic types without HPV16 and HPV18) > HPV66 (nontargeted carcinogenic type frequently detected by hc2) > HPV53, HPV67, HPV70, HPV82 (noncarcinogenic types frequently detected by hc2) > Other (other HPV genotypes).

Abbreviation: NA, sample was not tested for HPV genotypes.

cross-reactivity across many types that led to a similar frequency of cross-reactivity among single nontargeted types in this study as observed for hc2. Remarkably, when the Amplicor cutoff was increased to 1.5, there was an increase in analytic specificity for targeted HPV genotypes, with greater decrease in detection of nontargeted types than targeted types. Most of the nontargeted types frequently detected by hc2 (HPV53, HPV66, HPV67, HPV70, HPV82) were detected at much lower frequencies by Amplicor. Only HPV61 was more frequently detected by Amplicor at all cutoffs versus hc2.

The higher cross-reactivity of hc2 resulted in a higher detection of LSIL associated with noncarcinogenic types compared with Amplicor. This is consistent with a recent study of almost 6,000 women enrolled in the HPV Vaccine Trial in Costa Rica (CVT; ref. 37).

Although at the Amplicor 1.5 cutoff the sensitivity to detect CIN3+ was identical to hc2 in all study arms, there were apparent HPV genotype differences between the missed cases: hc2 more likely missed cases caused by carcinogenic types, including HPV16 and HPV18, whereas Amplicor was more likely to miss cases caused by nontargeted types (Table 4). We know from large cross-sectional surveys of HPV genotype prevalence in cervical cancers that most cases worldwide are caused by HPV16 and HPV18, fewer by other carcinogenic types, but only very few by noncarcinogenic types (38). This suggests that the nontargeted/noncarcinogenic types either do not progress to cancer at all, or that their progression takes such a long time that they are usually detected before invasive cancer occurs. In contrast, there is increasing evidence that women with cancers and CIN3 caused by HPV16 are younger than women with cancers and CIN3 caused by other types, suggesting that HPV16-associated progression is faster than that of lesions caused by other types (39). Therefore, despite the similar sensitivity between Amplicor and hc2, the qualitative difference between the two assays could be important—it might be more acceptable to miss disease caused by a low-risk type than if it was caused by HPV16 or HPV18. Currently, we cannot discriminate between CIN3 lesions with lower versus higher risk of invasion, but it is becoming increasingly clear that there is considerable heterogeneity in this disease category (3). Whereas the current histology-based end points cannot address the potential misclassification of precancers, new molecular markers might improve the risk assessment and offer new approaches of cervical cancer screening, e.g., using a sensitive HPV detection assay as a primary screen and a sensitive but highly disease-specific marker to determine the progression risk of HPV-positive women (40).

In summary, despite the higher analytic sensitivity and specificity of Amplicor (Fig. 1B), its clinical sensitivity was only marginally higher and its specificity was significantly lower than that of hc2 (Fig. 1A). Some CIN3+ cases positive for nontargeted types were missed by Amplicor but detected by hc2 due to the higher level of cross-reactivity. However, we currently do not know the relevance of detecting these cases, because progression to cancer seems to be very rare for lesions caused by nontargeted types. Although Amplicor detected more cases caused by targeted types due to its higher analytical sensitivity, the increased detection rate comes at the price of detecting many infections that do not seem

to be clinically relevant, at least in the young ALTS population and within the 2-year follow-up time period.

Finally, the performance of Amplicor at the 1.5 cutoff, which closely mimics the performance of hc2, needs to be pursued further. For example, a re-analysis of previous studies using higher cutoff values would be very simple and could yield results quickly. If our results are confirmed in different studies, using a higher cutoff value for Amplicor than currently recommended might be desirable.

Disclosure of Potential Conflicts of Interest

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