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Agreement between the AMPLICOR Human Papillomavirus Test and the Hybrid Capture 2 Assay in Detection of High-Risk Human Papillomavirus and Diagnosis of Biopsy-Confirmed High-Grade Cervical Disease▽

Francesca Carozzi,* Simonetta Bisanzi, Cristina Sani, Marco Zappa, Silvia Cecchini, Stefano Ciatto, and Massimo Confortini

Centro per lo Studio e la Prevenzione Oncologica, Analytical Cytology and BioMolecular Unit, Via Cosimo il Vecchio 2, 50127 Florence, Italy

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The AMPLICOR HPV test (AMP) and the Hybrid Capture 2 assay (HC2) detect 13 high-risk human papillomavirus (HR-HPV) types. Evaluation of comparative performance with clinical samples is needed to allow informed implementation of AMP into clinical practice. AMP was used (i) to assess the prevalence of HR-HPV in 1,032 samples of known cytology, HC2 status, and/or confirmed histology; (ii) to determine agreement between AMP and HC2; (iii) to evaluate the clinical sensitivity and specificity for detecting HR-HPV; and (iv) to detect the presence of biopsy-confirmed high-grade cervical intraepithelial neoplasia. The prevalence of HR-HPV was 39.3% and 45.6% by AMP and HC2, respectively. Overall agreement was 89.2% (kappa value, 0.78). Of 509 HR-HPV-negative specimens by HC2, 488 (95.9%) were AMP negative. Of 427 HR-HPV-positive specimens by HC2, 347 (81.2%) were AMP positive. In comparing the ability to detect high-grade squamous intraepithelial lesions (HSIL), the two tests were positive for all HSIL samples. Both tests performed similarly on CIN2+ samples (clinical sensitivities were 96.7% and 97.8%, respectively, for AMP and HC2). The clinical specificities of AMP and HC2 were comparable (54.9% versus 51.6%; $P = 0.18$). Genotyping of 20 HC2-negative/AMP-positive cases using alternative technologies revealed target HR genotypes in 63.1% of cases and low-risk types in 15.7% of cases, while 21% of cases were negative. In conclusion, AMP provides a viable alternative to HC2, with good agreement for samples with high-grade cytology and similar sensitivity in detecting CIN2+ lesions.

Infection with human papillomavirus (HPV) has been detected in 95 to 100% of cervical cancers, the second most common female cancer worldwide (30), and persistent infection with high-risk HPV (HR-HPV) types is known to be the first step in the process of carcinogenesis. Several epidemiologic and molecular studies suggest the use of HPV testing in order to improve the efficacy of population-based screening programs for cervical cancer. HPV testing has been found to be useful for triaging minor cytological abnormalities and in the follow-up of treated cervical intraepithelial neoplasia (CIN) (2, 4, 7, 27). HR-HPV DNA testing has been shown to be more sensitive than cytology and may improve patient management when used in addition to cytology (3, 4, 11); such testing is also under investigation as a primary screening tool (1, 10, 26). The negative predictive value of HR-HPV testing is very high (99.0%), which may allow screening intervals to be increased for women found to be negative by both cytology and HPV testing (16).

The Digene Hybrid Capture 2 assay (HC2) (Digene, Gaithersburg, MD) is commonly used for HPV testing to detect 13 common HR-HPV types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). Recently, the Roche AMPLICOR HPV test (AMP) (Roche Molecular Systems, CA) was made available for the same purpose. AMP detects the same 13 HR-HPV types detected by HC2 and, moreover, uses amplification of the β-globin gene as an internal measure of sample integrity and adequacy. Since it is a PCR-based method, the assay can be performed on small aliquots of samples in liquid cytology media or on samples obtained from archival paraffin-embedded tissue, and it is extremely sensitive (detects <100 copies of HPV DNA/PCR). In addition, incorporation of AmpErase (uracil-*N*-glycosylase [UNG]) into the master mix allows selective destruction of carryover products (containing deoxyuridine) from previous amplification reactions. However, evaluation of both analytical and clinical sensitivity is needed to support the use of AMP in clinical practice and cervical cancer screening programs.

In the present study, we assessed the prevalence of HR-HPV in samples from subjects with cytological abnormalities (atypical squamous cells of undetermined significance [ASC-US], atypical glandular cells of undetermined significance [AGC-US], low-grade squamous intraepithelial lesions [LSIL], and high-grade squamous intraepithelial lesions [HSIL]) as well as in samples from women with normal cytology; we also evaluated the analytical agreement between AMP and HC2 in detecting HR-HPV, and for analytical determinations, genotyping was carried out on discordant samples. In addition, the clinical sensitivity and specificity of AMP for histologically

* Corresponding author. Mailing address: Centro per lo Studio e la Prevenzione Oncologica, Unità Operativa Citologia Analitica e Molecolare, Via Cosimo il Vecchio 2, 50127 Firenze, Italy. Phone: 055-32697852. Fax: 055-32697879. E-mail: f.carozzi@cspo.it.

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TABLE 1. Overall agreement and specific agreement of HC2 and AMPLICOR HR-HPV tests by HC2 signal intensity ratio

HR HC2 result (signal intensity [RLU/CO])	*n*	No. of samples with AMP result		% Agreement
		Negative	Positive	
Negative (<1)	509	488	21	95.8
Positive				
Overall (≥1)	427	80	347	81.2
≥1 to 5	82	39	43	52.4
>5 to 10	23	10	13	56.5
>10 to 20	32	9	23	81.8
>20	290	22	268	92.4

TABLE 2. HPV prevalence in cervical specimens by cytological diagnosis and final outcome: comparison between the AMPLICOR HPV Test and HC2 assay

Diagnosis or final outcome	No. (%) of positive samples		Total no. of cases
	HC2	AMPLICOR	
Cytological diagnoses (n = 930)			
Negative	252 (38.1)	201 (30.5)	660
ASC-US/AGC-US	53 (36.5)	48 (33.1)	145
LSIL	47 (90.4)	45 (86.5)	52
HSIL	73 (100)	73 (100)	73
Final outcomes (n = 416)			
Negative	165 (67.6)	124 (50.8)	244
CIN1	62 (78.4)	57 (72.1)	79
CIN2+	91 (97.8)	90 (96.7)	93

confirmed >CIN2+ lesions were compared to those for disease detection with HC2. Finally, we calculated the clinical agreement for detecting underlying high-grade cervical disease for both AMP and HC2.

MATERIALS AND METHODS

This study was based on 1,032 cervical specimens collected in the Florence Cervical Cancer Screening Program, which regularly invited all resident women aged 25 to 64 years.

Of these, 962 samples were collected in Florence, Italy, within a study evaluating new technologies for cervical cancer screening, including HC2 HPV testing (the study was approved by the local ethical committee, implying informed written consent). In order to increase the prevalence of CIN2+ cases in the studied series, 70 samples from subjects with histologically confirmed CIN2+ were also included. These patients were referred to the colposcopy clinic at the Centro per lo Studio e la Prevenzione Oncologica of Florence after detection of abnormal cytology or for follow-up after conservative treatment of CIN (samples were taken at the time of colposcopy for study purposes only, implying written informed consent).

At the time of collection, samples were processed for liquid-based cytology and HC2 testing; samples were then stored at room temperature.

Cytological diagnoses were reported according to the Bethesda System 1991, namely, negative, ASC-US/AGC-US, LSIL, HSIL, invasive squamous cervical carcinoma, and invasive adenocarcinoma. The cytological diagnoses for the 1,032 samples were inadequate specimen (n = 7), negative (n = 744), ASC-US/AGC-US (n = 151), LSIL (n = 56), and HSIL (n = 74).

HPV testing by HC2 was performed after cytology, using 4 ml of PreservCyt medium (Cytyc Corporation, Marlborough, MA) for each sample and a sample conversion kit (Digene, Gaithersburg, MD) according to the manufacturer's instructions. HC2 was performed using only probe set B for HR-HPV types. The recommended positivity threshold of 1 pg/ml was used as a cutoff, and all samples with a relative light units/control (RLU/CO) ratio of ≥1.00 were considered positive.

Women with ASC-US or SIL and those aged 35 years or more and positive by HC2 with any cytology (including negative) were referred for immediate colposcopy. For HPV-positive women of <35 years of age with negative cytology, colposcopy was performed if HPV positivity persisted after 1 year.

For study purposes, histological diagnosis upon colposcopy-directed punch biopsy or diagnostic loop resection was taken as the gold standard for the presence of CIN2+. In terms of correlation between the two tests, all cases were considered. To avoid bias in the estimation of clinical sensitivity and specificity, only those 270 cases resulting from Pap test-positive specimens were selected.

AMPLICOR HPV test. HR-HPV detection using AMP was performed on stored samples by following the manufacturer's instructions. The positive cutoff point for AMP was an A_{450} value of 0.2.

AMP results were compared with cytology and histology status and also with HC2 results, stratified by HC2 signal intensity.

Genotyping assays. Samples with discrepant HR-HPV results (either positive by HC2 and negative by AMP or negative by HC2 and positive by AMP) were genotyped for the presence of HPV, using two different PCR-based assays. First, discrepant samples were tested with the Roche Linear Array (LA) test to detect a total of 37 high- and low-risk (LR) types (14, 15). If HR-HPV was not detected using the Roche LA test, then another PCR-based assay was used to detect HR-HPV with E6/E7 primers, as previously described (30).

For the Roche LA test, 50 μl of extracted DNA (the same DNA used for AMP) was added to 50 μl of working master mix, following the manufacturer's instructions. The LA detect 37 HR- and LR-HPV genotypes, namely, types 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51 to 56, 58, 59, 61, 62, 64, 66 to 70 (CPT141 and LVX180), 71 (CP8081), 72, 73 (Pap238A and MM9), 81 (CP8304), 82, IS39, 83 (P291 and MM7), 84 (P155 and MM8), and 89 (CP 6108).

HPV types considered high risk for the purpose of this study were types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 (8, 20). All other types were considered low risk.

The E6/E7 PCR assay is a highly sensitive gold standard method for detecting HR-HPV genotypes and is based on that previously described by Walboomers et al. (30). Samples for this assay were extracted from 2.5 ml of stored PreservCyt solution by using a QIAamp tissue kit (QIAGEN) according to the manufacturer's instructions. The genotypes detected with the E6/E7 primer system are the 13 HR-HPV types detected by HC2 and AMP, as well as types 53 and 66, which have previously been shown to generate positive results with HC2 analysis (21, 29).

Statistical analysis. To estimate clinical sensitivity and specificity, we defined all histologically confirmed CIN2+ samples as true lesions and all other specimens as truly negative. McNemar's chi-square (χ^2) test was applied (18). As discussed, the analysis was restricted to Pap test-positive specimens.

The agreement between AMP and HC2 was evaluated by means of the K statistic proposed by Cohen (9). According to the statistical literature, K values of <0.40 usually indicate poor agreement, values from 0.40 to 0.75 indicate fair to good agreement, and values of >0.75 indicate excellent agreement.

RESULTS

HPV prevalence by AMP and HC2. AMP was performed on samples stored for 2 years at room temperature: 96/1,032 samples (9.3%) tested β-globin negative at this time point and were excluded from the study. Of the remaining 936 valid samples, AMP was positive for 368 (39.3%) and HC2 was positive for 427 (45.6%) (Table 1). The overall agreement between the two tests was 89.2%, with a kappa value of 0.78. Of the 509 specimens that were negative by HC2, AMP was negative for 488 (95.9%). Among 427 specimens that were positive by HC2, AMP was positive for 347 (81.2%).

Agreement between AMP and HC2, stratified by HC2 signal intensity, is shown in Table 1. A significant association of agreement and HC2 signal intensity was evident (χ^2 for trend, 77.3; $P = 10^{-6}$).

HPV prevalence by AMP and HC2 compared to that by cytology and histology. Table 2 shows the prevalence of HPV according to cytological diagnosis in 930 samples (6 samples were excluded because of inadequate cytology) and the final outcomes for 416 women who underwent, according to the

TABLE 3. Cytology and histology results in discordant samples that were positive by HC2 and negative by AMPLICOR

Histology	Cytology result (no. of samples)					Total no. of cases
	Negative	ASC-US/ AGC-US	LSIL	HSIL	Inadequate specimen	
Negative	61	8	3	0	2	74
CIN1	3	2	0	0	0	5
CIN2-3	0	1	0	0	0	1
Total	64	11	3	0	2	80

study protocol, colposcopy and/or biopsy. All 73 HSIL samples were HPV positive with both tests. Of 79 CIN1 histological lesions, 57 were positive by AMP (72.1%) and 62 were positive by HC2 (78.4%). Of 93 CIN2+ lesions, 90 (96.7%) were positive by AMP and 91 (97.8%) were positive by HC2.

Clinical sensitivity and specificity. For estimating clinical sensitivity and specificity, we selected only the 270 Pap test-positive cases (ASC-US or SIL), including 86 confirmed CIN2+ lesions and 184 truly negative samples (no lesion or lesions less severe than CIN2+). In considering the 86 CIN2+ lesions, HC2 identified one more lesion (84/86 [97.7%]) than did AMP (83/86 [96.5%]). However, the difference in sensitivity was not statistically significant [McNemar's $\chi^2(1) = 1.00$; $P = 0.3173$]. Also, the specificity of AMP (101/184 [54.9%]) was not significantly different from the specificity of HC2 (95/184 [51.6%]) [McNemar's $\chi^2(1) = 1.80$; $P = 0.1797$].

Table 3 shows that the majority of results that were negative by AMP and positive by HC2 corresponded to both cytology- and histology-negative results (61/80 samples [76.2%]). Abnormal cytology was present in some samples that were negative by AMP and positive by HC2, including 11/80 (13.7%) ASC-US/AGC-US samples and 3/80 (3.7%) LSIL samples. With regard to histology, CIN1 was confirmed in 5/80 samples (6.2%) and CIN3 was confirmed in 1 sample (1.2%). Follow-up data were available for 60/80 cases (75%), and no additional CIN2+ cases were recorded.

Among 21 patients with negative HC2 results and positive AMP results, both cytology and histology results were negative in 13/21 (61.9%) cases. Abnormal cytology was detected in some samples that were negative by HC2 and positive by AMP, including ASC-US (7/21 [33.3%]) and LSIL (1/21 [4.7%]), and one case was inadequate. CIN1 histology was confirmed in one case (1/21 [4.7%]).

Results of genotyping analysis of discordant samples. The aim of this study was to assess the agreement of HC2 and AMPLICOR, which are both designed to reveal the same 13 high-risk HPV types. For the discordant samples (either positive by HC2 and negative by AMP or negative by HC2 and positive by AMP), further genotyping was aimed at assessing the presence of high-risk types, that is, determining if AMP results were falsely negative, HC2 results were falsely positive, or vice versa.

Cases with discordant results were investigated by genotyping, using two methods. The LA assay was performed on 78/80 cases that were positive by HC2 and negative by AMP (two samples were excluded because of insufficient DNA). HPV genotypes were identified in 36/78 samples. High-risk target genotypes were identified in seven cases, of which four showed coinfection with LR genotypes. In total, LR genotypes were observed in 25 cases, of which 7 cases showed coinfections with other LR genotypes. A nontarget HR genotype (HPV73) was observed in four cases, all of which showed coinfection with LR genotypes. The 42 samples that tested HPV negative using the LA assay were tested further with E6/E7 primers. Four samples were not analyzed by E6/E7 primers because of insufficient DNA. Twenty-two of these 42 samples were identified as containing HR-HPV target genotypes by the E6/E7 assay, 2 samples were positive for HPV53, 1 was positive for HPV66, and 13 tested negative.

TABLE 4. Final HPV genotyping (combined results by Roche LA test and type-specific PCR for E7 open reading frames) and cytology results for 80 discordant specimens (positive by HC2 and negative by AMPLICOR)

Genotype[a]	No. of specimens	Cytology result (no. of specimens)
16	2	Negative (1), ASC-US (1)
18	6	Negative (5), ASC-US (1)
31	8	Negative (7), ASC-US (1)
33	2	Negative (2)
39	4	Negative (4)
42	2	Negative (2)
45	1	Negative (1)
53	7	Negative (5), ASC-US (2)
54	3	Negative (2), ASC-US (1)
58	2	Negative (2)
61	2	ASC-US (2)
62	2	Negative (1), ASC-US (1)
66	3	Negative (3)
70	1	Negative (1)
42 + 53	1	LSIL (1)
45 + 61	1	Inadequate (1)
55 + 66	1	Negative (1)
53 + 73	1	Negative (1)
62 + 66	1	Negative (1)
73 + 62	1	ASC-US (1)
53 + 84	1	LSIL (1)
84 + **58**	1	Negative (1)
55 + 53 + **58**	1	Negative (1)
6 + 82 + **51** + 42	1	Negative (1)
66 + 73 + 84 + 42 + 54	1	LSIL (1)
53 + 81 + 71	1	Negative (1)
62 + 73 + IS39 + 40	1	Negative (1)
66 + cp6108	2	Negative (2)
Cp6108	1	Negative (1)
Not typed	13	Negative (11), inadequate (1), ASC-US (1)
No sample available	6	Negative (6)
Total	80	80

[a] High-risk target genotypes are shown in bold.

Table 4 summarizes the final results obtained for the 80 cases that were positive by HC2 and negative by AMP and were analyzed with the two genotyping methods, together with cytological findings. Genotypes were identified for 61/74 samples (82.4%). Of these, 25 cases (40.9%) contained a single HR-HPV genotype, 8 (13.1%) contained HR and LR genotypes, and 28 (45.9%) contained single or multiple LR genotypes. Of 12 samples that were positive by HC2 with negative typing, 11 had low (<15) HC2 RLU/CO ratios (data not shown).

Genotyping with the LA assay was also performed on 20/21

TABLE 5. Final HPV genotyping (combined results by Roche LA test and type-specific PCR for E7 open reading frames) and cytology results for 21 discordant specimens (negative by HC2 and positive by AMPLICOR)

Genotype[a]	No. of specimens	Cytology result (no. of specimens)
16	1	Negative (1)
31	1	Negative (1)
45	1	Negative (1)
51	3	Negative (3)
53	2	ASC-US (2)
56	1	LSIL (1)
61	1	Negative (1)
16 + 59	1	Negative (1)
18 + 52	1	ASC-US (1)
35 + 52	1	ASC-US (1)
59 + 61	1	Negative (1)
31 + 42 + 53 + CP6108	1	Negative (1)
Not typed	4	Negative (3), inadequate (1)
No sample available	2	ASC-US (2)
Total	21	21

[a] High-risk target genotypes are shown in bold.

samples that were negative by HC2 and positive by AMP (1 sample was excluded because of insufficient DNA). Infection with only target HR genotypes was observed in seven cases; two samples had HR- and LR-HPV coinfections, and two cases showed only LR genotypes. In total, eight of nine LA-negative samples underwent E6/E7 typing (one sample was not analyzed because of insufficient DNA). Four samples tested E6/E7 negative, three samples showed HR target genotypes, and one showed HPV53.

Table 5 summarizes the final results obtained for 21 cases that were negative by HC2 and positive by AMP and were analyzed with the two genotyping methods, together with cytological findings. For two cases, no sample was available. Genotypes were identified in 15/19 samples (78.9%): 12/15 (80%) contained HR-HPV genotypes and 3/15 (20%) contained only LR-HPV genotypes.

As shown in Tables 4 and 5, the two assays demonstrated slightly different profiles for the detection of particular genotypes within discrepant samples. Both assays showed some variation in the ability to detect target genotypes 16, 18, and 31 and nontarget genotypes 53 and 61, while HC2 showed a tendency to give a positive test result in the presence of nontarget genotype 66. It is worth mentioning that HPV types 53 and 66 are assumed to be probable high-risk types (20), whereas HPV61 is assumed to be a low-risk type.

DISCUSSION

The aim of the present study was to compare AMP, a new, commercially available assay, to HC2 in detecting HR-HPV in 1,032 cervical samples. AMP requires small amounts of material and can therefore be carried out on archival samples, although prolonged storage may reduce the number of archived samples that can be amplified successfully. The proportion of samples that failed to amplify after storage in our study was similar to previously observed failure rates for archived liquid-based cytology samples after several years of storage (6).

Two recent studies evaluated the analytical performance of the newly introduced AMPLICOR HPV test. Poljak et al. (22) obtained concordant results between HC2 and AMP in 85.9% of tested samples. van Ham et al. (28) compared another commercial PCR-based assay, SPF10-LiPA (Innogenetics, Belgium), with AMP and found absolute agreement (97.5%) between the two tests. However, these studies did not correlate HPV testing results with histological findings, as they used only cytological findings. Recently, Sandri et al. (25) compared the performances of AMP and HC2 for women attending a colposcopy clinic either for follow-up or for clinical purposes, and they found concordant results for 83% of the samples.

The overall agreement in our study is comparable with the values obtained in the above-mentioned studies, and moreover, we observed that agreement between AMP and HC2 varied with the HC2 signal intensity, with the lowest agreement being observed for samples with low HC2 signal intensities.

HPV testing performance should most appropriately be based on its clinical sensitivity and specificity for CIN2+ lesions, particularly if HPV testing is going to be employed in a screening setting, alone or in combination with the Pap test.

Monsonego et al. (19) tested AMP performance for women with abnormal Pap tests or attending a clinic for opportunistic infection screening, and they showed AMP to be highly sensitive (95.2%) in detecting CIN2+ lesions. In this study, however, only women with abnormal Pap tests had colposcopy, whereas the primary screening group was checked on a liquid-based cytology basis.

Our study was aimed at assessing whether HPV testing has a higher accuracy for detecting CIN2+ than that of cytology, and in order to increase its power, it was designed to include a very large number of colposcopy- and histology-confirmed lesions.

The reproducibility of histological diagnosis in reporting cervical intraepithelial neoplasia may be suboptimal (17), particularly as far as CIN1 is concerned (12), but this consideration also applies to the reproducibility of cytological diagnosis. In order to minimize this effect, a subset of histology-confirmed lesions were independently reviewed in a blinded fashion, as already described (24). The two tests performed similarly in the detection of confirmed CIN2+ lesions, and even though HC2 identified one more lesion than AMP, clinical sensitivities were not statistically significantly different between the two tests. At the same time, AMP specificity was higher, but the difference was not statistically significant.

It is worth noting that in the present study, cases were selected partially on the basis of their HC2 test-positive (and cytology-negative) status, a condition that would bias HC2 performance towards higher sensitivity and lower specificity. Nevertheless, when the analysis was repeated on cytology-positive-only cases, results were similar to those observed for the whole series. Moreover, AMP clinical sensitivity might have been underestimated due to the retrospective study design, which excluded from analysis patients whose samples were negative by cytology and HC2, who were not referred for colposcopy but might still harbor a slight proportion of potential AMP test-positive CIN2+ lesions.

Criteria to define the reference standard for the presence of

HPV infection may be a problem in our study and other similar studies; when evaluating the analytical sensitivities of the two methods (AMP and HC2), samples with negative or positive results by both methods were assumed to be truly negative or truly positive, whereas discordant samples were tested with two different alternative technologies.

Discordant results between the two tests were found in 10.8% of samples. Genotyping with two different methods to confirm the presence of HR-HPV showed a relatively high frequency (45.9%) of LR-HPV types among samples that were positive by HC2 and negative by AMP, a finding consistent with previous reports of HC2 cross-reactions with LR-HPV types (5, 23); all LR-HPV types observed in the present series had already been described as cross-reactive. A low frequency (15.7%) of LR types was identified among samples that were negative by HC2 and positive by AMP. Poljak et al. (22) genotyped all samples tested with HC2 and AMP, and LR types were found in 4 of 862 samples. Sandri et al. (25) genotyped discordant samples and found LR types in 30% of samples testing negative by HC2 and positive by AMP. The different findings between the study of Poljak et al. (22) and the studies presented here and by Sandri et al. (25) might be ascribed to the typing methods used (LA and Inno-LiPA, respectively), where LA detects 37 genotypes and Inno-LiPA detects 27 types. Our study, like that performed by Sandri et al. (25), is based, however, on a small number of typed samples, and no other reports are available on this issue to our knowledge. Considering the possibility that genotypes other than those included in the panel may cross-react with AMP, larger studies are probably needed to clarify this issue.

It is likely that prophylactic HPV vaccines will be available in the near future, and it has been emphasized that in such a scenario it would be important to distinguish between single HPV types rather than simply detecting the presence of HR-HPV by using pooled probes. In particular, it will be important to detect the presence of the specific type to which the vaccine is aimed and to verify to what extent a vaccination program may alter the type-specific HPV prevalence over time (13).

For such purposes, AMP or HC2, when coupled with genotyping, might give more detailed information on incident HPV infections.

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