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Comparison of the Digene HC2 Assay and the Roche AMPLICOR Human Papillomavirus (HPV) Test for Detection of High-Risk HPV Genotypes in Cervical Samples

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Many different methods with different sensitivity and specificity have been proposed to detect the presence of high-risk human papillomavirus (HR HPV) in cervical samples. The HC2 is one of the most widely used. Recently, a new standardized PCR-based method, the AMPLICOR HPV test, has been introduced. Both assays recognize the same 13 HR HPV genotypes. The performances of these two commercially available assays were compared in 167 consecutive women (for a total of 168 samples) who presented at the Colposcopy Clinic either for a follow-up or for a diagnostic visit. Concordant results were found in 140/168 cervical samples (overall agreement, 83%; Cohen's kappa = 0.63). Twenty-eight samples gave discordant results: 20 were positive with the AMPLICOR HPV test and negative with the HC2 assay, and 8 were negative with the AMPLICOR HPV test and positive with the HC2 assay. The genotyping showed that no HR HPV was detected in the 8 HC2 assay-positive AMPLICOR HPV test-negative samples, while in 8/20 AMPLICOR HPV test-positive HC2 assay-negative samples, an HR HPV genotype was found. The AMPLICOR HPV test scored positive in a significantly higher percentage of subjects with normal Pap smears. All 7 cervical intraepithelial neoplasia grade 3 patients scored positive with the AMPLICOR HPV test, while 2 of them scored negative with HC2. Both tests had positive results in the only patient with squamous cell carcinoma. In conclusion, this study shows that the HC2 assay and the AMPLICOR HPV test give comparable results, with both being suitable for routine use. The differences noted in some cases may suggest a different optimal clinical use.

A causal link between human papillomavirus (HPV) infection and cervical cancer has been well established (2, 12, 14, 23). A large number of HPV genotypes have been identified, and the mucosal HPV strains are divided into "high-risk" (HR) and "low-risk" (LR) categories on the basis of their association with cervical lesions. The HR types are more frequently found in premalignant or malignant lesions, LR types are found in benign lesions such as condylomata acuminata (18). Infection by HR HPV types has been demonstrated in almost 100% of cervical carcinoma (29), and it has been recently shown that persistent infection with the same genotype strongly increases the risk of developing high-grade preinvasive disease (11).

The detection of HR HPV in cervical samples has been proposed to improve the efficacy of cervical carcinoma screening programs and to triage women with ambiguous or borderline cervical smears (1). Women with persistent HR HPV positivity have a clearly enhanced risk of developing a premalignant lesion and, hence, may be more closely monitored (3, 15). Moreover, HR HPV testing may be associated with Pap smear to monitor women who have been treated for high-grade cervical intraepithelial neoplasia (CIN) (16, 30). Several studies have revealed that HPV testing yields a high negative predictive value, approaching 100%, for high-grade CIN lesions and cervical carcinomas (lesions ≥ CIN3) (21, 22, 31).

Testing for HPV relies on the detection of viral DNA. The only test currently approved by the U.S. Food and Drug Administration for the detection of HPV DNA is the Hybrid Capture 2 (HC2) system (Digene Corporation, Gaithersburg, Md.). The HC2 assay is a ready-to-use test for routine diagnostics and uses a liquid hybridization format followed by signal amplification to detect 13 HR HPV types (i.e., genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) by means of an RNA cocktail probe; the test does not distinguish individual HPV types. The sensitivity is approximately 4,700 viral copies/ml of cervical sample suspension. The evaluation of its laboratory performance has shown that HC2 is a reliable and reproducible test (4, 6, 8); both characteristics are fundamental for a test with potential widespread use. Different laboratories have also used many PCR-based methods. PCR methods are considered the "gold standard" for analytical sensitivity to detect infectious organisms, including HPV. However, PCRs for HPV detection are currently performed as "home brew" methods, lack standardization, show different sensitivity and specificity, are time-consuming, and require a demanding job for the laboratory. In the last years, some studies have compared the performance of HC2 and PCR, showing a good level of agreement between the two methods (19, 28).

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Recently, a standardized PCR-based technique (AMPLICOR HPV test; Roche Molecular Systems) for the detection of the 13 HR HPV genotypes has been commercialized. This new test uses amplification of target DNA by PCR and nucleic acid hybridization for the detection of HR HPV genotypes in cervical cells collected into a transport medium. This test could be used in clinical diagnostic laboratories, but few data are currently available on its performance in a clinical setting (17, 27) and, in particular, no data exist on its comparison with the HC2 assay.

The purpose of this study was to examine the performance of two commercially available assays, the HC2 assay and the AMPLICOR HPV test, to detect the presence of HR HPV in cervical samples of women who attended the Colposcopy Clinic for an evaluation of an abnormal Pap smear or for a follow-up examination after a CIN treatment. This analysis will be useful to better identify the assay more suitable for a particular application as well as to inform the clinicians about the correct interpretation of the results of any assay.

MATERIALS AND METHODS

Study population. One hundred sixty-seven consecutive women attending the Colposcopy Clinic of the European Institute of Oncology (Milan, Italy) from July to September 2004 were prospectively enrolled. The cohort consisted of women who presented (i) for screening purposes, (ii) for evaluation of an abnormal Pap smear, or (iii) for a follow-up visit after a conservative treatment. Specimens for the HC2 assay and AMPLICOR HPV test were collected from all women during the visit. In case of discrepant results between HC2 assay and AMPLICOR HPV test, genotyping was performed. Moreover, Pap smears were available for 162 subjects. Colposcopy was performed when necessary, and a histological sample was taken if a lesion was identified. The median age of the women was 40 years (range, 21 to 69 years). Written consent was obtained from all subjects.

Sample collection. Cervical samples were taken using a Cervex brush (Rovers Medical Devices B.V., Holland). The collected specimen was first smeared on a glass for conventional cytological evaluation, and then the brush was washed in a vial containing PreservCyt solution (Cytyc Corporation, Boxborough, MA) and transferred to the laboratory for HPV analyses (H. A. Cubie, K. S. Cuscheri, and C. Moore, 21st Int. Papillomavir. Conf., abstr. 121, 2004). The HC2 assay was performed weekly. A 500-μl aliquot of each sample was removed and frozen at −80°C for DNA extraction and subsequent testing for HPV DNA by PCR. In case of discrepant results, the specimen was genotyped using the LINEAR ARRAY HPV genotyping test (Roche Molecular Systems).

HC2 HR HPV assay. Sample material collected in PreservCyt medium was made suitable for HC2 assay using a sample conversion kit (Digene Corporation). HPV DNA testing by the HC2 assay method was performed with the automated HC2 assay system according to the manufacturer's protocol. HC2 is a sandwich capture molecular hybridization assay that utilizes chemiluminescent detection to provide a semiquantitative result. The assay is calibrated to detect approximately 4,700 genome equivalents (or 1 pg/ml) of target HPV, represented by an RLU (relative light unit) measurement greater than or equal to the cutoff value calculated in each run by a series of standards. A measurement less than the cutoff was scored as negative. The samples were analyzed for the presence of HR HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. Positive and negative controls (provided by the manufacturer) were included in each run.

AMPLICOR HPV test. (i) DNA extraction. DNA, both HPV and cellular, was released by lysing cervical specimens under denaturing conditions at elevated temperatures in the presence of proteinase K. DNA purification was obtained in columns with a silica-based membrane using vacuum processing.

(ii) PCR amplification. The AMPLICOR HPV test uses biotinylated primers to define a sequence of approximately 165 bp in length within the polymorphic L1 region of the HPV genome. The primers, pooled in the same PCR master mix, are designed to amplify viral DNA from the same 13 types included in the HC2 assay. Also with the same master mix, the β-globin gene was amplified (268 bp amplicon) to test whether the extracted DNA would be suitable for use as a template for PCR.

(iii) Hybridization reaction. Capture probes, representing regions internal to the amplified sequences, were used to identify the viral or human DNA. Following PCR amplification, the amplicons were chemically denatured to form single-stranded DNA and added to separated wells of microwell plates (MWP) coated with either HR HPV probes or β-globin-specific oligonucleotide probe. The hybridization was done at 37°C for 1 h.

(iv) Detection reaction. Following the hybridization reaction, the MWP was washed to remove unbound material, and an avidin-horseradish peroxidase conjugate was added to each well to link the biotin. The MWP was washed again to remove unbound conjugate, and a substrate solution containing hydrogen peroxide and 3,3′,5,5′-tetramethyl benzidine (TMB) as a chromogen was added. The colorimetric reaction was done for 10 min at room temperature in the dark and stopped by the addition of a weak acid. The absorbance at 450 nm was measured immediately using an automated microwell plate reader.

According to the manufacturer's specifications, the AMPLICOR HPV test could detect genotypes 16, 18, 33, 35, 39, 45, 51, 56, and 68 at 100 copies/ml and genotypes 31, 52, 58, and 59 at 240 copies/ml, with a positivity rate greater than 95%. All genotypes are detected with a 100% positivity rate at 480 copies/ml.

LINEAR ARRAY HPV genotyping test. (i) DNA extraction. The specimen preparation was the same as in the case of the AMPLICOR HPV test.

(ii) PCR amplification. The LINEAR ARRAY HPV genotyping test uses biotinylated primers to define a sequence of nucleotides within the polymorphic L1 region of the HPV genome that is approximately 450 bp long. A pool of HPV primers is designed to amplify HPV DNA from 37 genotypes including 13 HR HPV genotypes (6, 11, **16**, **18**, 26, **31**, **33**, **35**, **39**, 40, 42, **45**, **51**, **52**, 53, 54, 55, **56**, **58**, **59**, 61, 52, 64, 66, 67, **68**, 69, 70, 71, 72, 73, 81, 82, 83, 84, CP6108, and IS39; HR HPV genotypes are indicated in boldface type). Capture probe sequences are located in polymorphic regions of L1 bound by these primers. An additional primer pair targets the human β-globin gene. Ampli*Taq* Gold DNA polymerase is utilized for amplification of the HPV target DNA and the β-globin control.

(iii) Hybridization reaction. Following PCR amplification, the HPV and the β-globin amplicon were chemically denatured to form single-stranded DNA by the addition of denaturation solution. Aliquots of denatured amplicon were then transferred to the appropriate well of the typing tray that contained hybridization buffer and a single LINEAR ARRAY HPV genotyping strip that was coated with HPV and β-globin probe lines. The biotin-labeled amplicon hybridized to the oligonucleotide probes only if the amplicon contained the matching sequence of the complementary probe.

(iv) Detection reaction. Following the hybridization reaction, the LINEAR ARRAY HPV genotyping strip was washed to remove any unbound material. Streptavidin-horseradish peroxidase conjugate was then added to the strip. The streptavidin-horseradish peroxidase conjugate bound to the biotin-labeled amplicon hybridized to the oligonucleotide probes on the strip. The strip was washed to remove any unbound conjugate, and a substrate solution containing hydrogen peroxide and TMB was added to each strip. In the presence of hydrogen peroxide, the bound streptavidin-horseradish peroxidase catalyzed the oxidation of TMB to form a blue-colored complex, which precipitated at the probe positions where hybridization occurred. The LINEAR ARRAY HPV genotyping strip was then read visually by comparing the pattern of blue lines to the reference guide. The limit of detection is reported by the manufacturer for HPV genotypes 16, 18, 31, and 45. Genotypes 16, 18, and 45 were detected with a 100% positivity rate at 300, 3,000, and 900 copies/ml, respectively. Genotype 31 was detected with a 92% positivity rate at 3,000 copies/ml.

Statistical analysis. Sensitivity, specificity, and accuracy of the AMPLICOR HPV test and of the HC2 assay were determined against the presumed HPV status based on the combination of both tests and on genotyping results in the case of discordant findings. The 95% confidence intervals were calculated using the exact method. Agreement between the two tests was assessed by Cohen's kappa statistic, with values of 0.00 to 0.20 indicating poor agreement, 0.21 to 0.40 indicating fair agreement, 0.41 to 0.60 indicating moderate agreement, 0.61 to 0.80 indicating good agreement, and 0.81 to 1.00 indicating excellent agreement. Marginal homogeneity of the two tests was assessed by McNemar's test. All tests were two-sided, and a P value of <0.05 was considered significant.

RESULTS

Among the 167 women studied (one woman contributed two samples), HR HPV DNA testing was positive by HC2 assay in 32.1% (54 of 168 observations) of the subjects and by AMPLICOR HPV test in 39.3% (66 of 168 observations) of the subjects. The concordance between the 2 tests is shown in Table 1. The two tests gave concordant results for 46 positive samples and 94 negative samples, with an overall level of agreement of 83.3% (Cohen's kappa = 0.63). However, 28 samples gave

TABLE 1. Concordance between the results of the 168 samples tested by the HC2 assay and AMPLICOR HPV test[a]

HC2 assay result	No. of samples with AMPLICOR HPV test result:		Total
	Positive	Negative	
Positive	46	8	54
Negative	20	94	114
Total	66	102	168

[a] Cohen's kappa = 0.63; McNemar $P = 0.036$.

discordant results: 8 were positive by the HC2 assay but negative by the AMPLICOR HPV test, whereas 20 samples were AMPLICOR HPV test positive but HC2 assay negative ($P = 0.036$). The results of the LINEAR ARRAY HPV genotyping are shown in Table 2. In none of the HC2 assay-positive AMPLICOR HPV test-negative samples was an HR HPV type detected; in 4 samples, HPV genotype 66 was found; and in two samples, HPV genotype 6 was present. In 8 of the 20 samples that were HC2 assay negative and AMPLICOR HPV test positive, an HR HPV type was isolated; in 5 samples, an LR HPV type was found; and in 7 cases, no HPV type was found by the LINEAR ARRAY HPV test.

Assuming that samples positive by both tests had all high-risk genotype and that samples negative by both tests are all HPV negative or low risk, we can evaluate a "conditional" sensitivity and specificity for both tests (Table 3). The specificity is similar for both tests, while the AMPLICOR HPV test shows, as expected, a higher sensitivity than the HC2. Moreover, also in the situation in which all of the untyped specimens (by the LINEAR ARRAY) were false positives (by the AMPLICOR HPV test), the specificity of the AMPLICOR HPV test decreased slightly but was still comparable with that of the HC2 assay.

TABLE 2. Overview of discordant cases

Test results	Genotype(s)[a]	No. of specimens
AMPLICOR HPV test positive, HC2 assay negative	**16**	2
	31	1
	6 + **59**	1
	16 + 83	1
	39 + **45**	1
	52 + 54	1
	58 + 81	1
	53	1
	82	1
	6 + 71	1
	42 + 62	1
	11 + CP6108	1
	None found	7
Total		20
HC2 assay positive, AMPLICOR HPV test negative	6	1
	66	3
	81	1
	6 + 42 + 71	1
	62 + 66 + 73	1
	None found	1
Total		8

[a] HR HPV genotypes are indicated in boldface type.

TABLE 3. HC2 assay and AMPLICOR HPV test performance

Test and result	Both tests negative or LR genotype (*n*)	Both tests positive or HR genotype (*n*)	% Sensitivity or specificity (95% CI)[a]
AMPLICOR[b]			
Negative	101	0	100 (96–100)
Positive	5	54	95 (89–98)
HC2[b]			
Negative	99	8	85 (73–93)
Positive	7	46	93 (86–97)
AMPLICOR[c]			
Negative	102	0	100 (96–100)
Positive	12	54	89 (83–94)
HC2[c]			
Negative	106	8	85 (73–92)
Positive	8	46	93 (87–96)

[a] Sensitivity corresponds to negative test results; specificity corresponds to positive test results. 95% CI, 95% confidence interval.
[b] Excluding data from 8 patients with no genotype determined.
[c] Including data from 8 patients with no genotyping, assuming that all nontyped specimens had false positives with the AMPLICOR HPV test.

Table 4 shows the HPV detection related to cytological results. Overall HR HPV DNA prevalence increased in parallel with the increasing severity of Pap smear result. With the AMPLICOR HPV test, a significantly higher percentage of normal Pap smear samples tested positive than with the HC2 assay ($P = 0.0018$), while the two tests performed similarly on the abnormal Pap smear samples. Moreover, HR HPV was detected in 3 of 6 women for whom a cytological result was not available, with concordant results with both tests.

For 33 women, a histological sample was available. Figure 1 presents the percentage of positivity of the two tests in the detection of cervical lesions. One CIN2 patient was negative by the AMPLICOR HPV test but positive by the HC2 assay, and two CIN3 patients were positive by the AMPLICOR HPV test but negative by the HC2 assay. In the case of the CIN2 patient (HC2 assay positive), the genotype showed the presence of only LR HPV (HPV genotypes 6, 42, and 71). In the two CIN3 cases (AMPLICOR HPV test positive), HR HPV genotypes were present (HPV 45 plus HPV 39 and HPV 16, respectively). The only patient with squamous cell carcinoma (SCC) tested positive by both tests.

DISCUSSION

The aim of the present study was to compare the performance of two commercially available assays (the HC2 assay and the new AMPLICOR HPV test) for the detection of the 13 HR HPV DNA in a group of 167 women. The overall concordance of the two tests was 83.3%, with a Cohen's kappa value of 0.63, which indicated a good correlation. In general, the AMPLICOR HPV test scored more HR HPV-positive samples than the HC2 assay (39.3% and 32.1%, respectively). This difference in the positivity rate can be explained by the higher sensitivity of PCR methods in general and of the AMPLICOR HPV test in particular compared to the HC2 assay. In fact, the two methods cover the same panel of

TABLE 4. Results of cervical cytology related to HPV DNA detection with HC2 assay and AMPLICOR HPV test

Pap smear result	No. of samples	No. of samples with AMPLICOR result				% Concordant	McNemar *P* value
		Negative		Positive			
		HC2 negative	HC2 positive	HC2 negative	HC2 positive		
Negative	96	69	1	13	13	85	0.0018
Abnormal[a]	66	22	7	7	30	79	1.00
ASCUS	24	13	3	3	5	75	1.00
LSIL	26	7	3	1	15	85	0.63
HSIL	16	2	1	3	10	75	0.63
Not available	6	3	0	0	3	100	
Total	168	94	8	20	46		0.036

[a] ASCUS, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion.

HPV genotypes; hence, the difference could not be ascribed to this reason.

Two recent studies evaluated the performance of the newly introduced AMPLICOR HPV test. The study by Monsonego et al. (17) assessed its performance both in the detection of cervical pathology in women with an abnormal Pap smear and in addition to the Pap smear in the screening setting. In this study, no other method for HPV detection was used, and the authors found that, together with colposcopy and high-grade squamous intraepithelial lesion cytology, the AMPLICOR HPV test is a powerful independent predictor of high-grade CIN2 and 3. The other study compared the performance of the AMPLICOR HPV test with that of the INNO-LIPA HPV detection/genotyping assay (27): the authors found the two tests to be fairly well correlated, with an absolute agreement between the two tests of 97.5%.

Instead, we found discordant results in 17% of the samples. The genotyping showed that, in all cases of HC2 assay-positive and AMPLICOR HPV test-negative samples, no HR HPV could be detected, but in 7 of 8 cases, one or more LR HPV type has been isolated. Recent studies have demonstrated that the Digene HC2 HR assay may have cross-reactivity with LR HPV types (5, 20), known to cause some cytological abnormalities, which never progress to cancer. All of the LR HPV types present in these 7 specimens had already been described as cross-reactive. In particular, in 4 samples, HPV genotype 66 has been isolated. Although this could be considered an advantageous cross-reactivity, due to the fact that HPV genotype 66 has recently been added to the HR HPV group (18), these cross-reactions can nevertheless lead to false-positive results, affecting patient management.

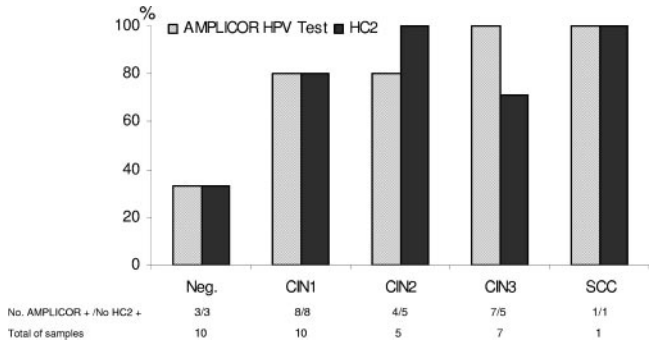

FIG. 1. Association between histological results and HPV testing. Both the HC2 assay and the AMPLICOR HPV test identified the patient with SCC, the AMPLICOR HPV test missed 1/5 CIN2 patients, and the HC2 assay missed 2/7 CIN3 subjects. On the figure, the percentages of HPV test positivity are reported according to histological result. Neg., negative.

Among the 20 samples which were AMPLICOR HPV test positive and HC2 assay negative, an HR HPV genotype was detected in 8 samples. In 7 of the remaining specimens, no HPV type was found: this could be due to either a low viral load (the AMPLICOR HPV test has a higher sensitivity than LINEAR ARRAY HPV genotyping) or a false-positive result due to the presence of contaminants in the sample. Assuming the worst situation, in which all untyped specimens could have been false positives by the AMPLICOR HPV test, the specificity of the AMPLICOR HPV test decreased slightly but was still comparable to that of the HC2 assay. In 5 cases, LR HPV genotypes were detected. To date, there are no reports evaluating the possibility of cross-reactivity with genotypes other than the 13 included in the panel. Larger studies are probably needed to clarify this issue.

The two tests gave substantially concordant results. However, in women with normal smears there was a difference in the positivity rate between the two assays. The AMPLICOR HPV test gave a significantly higher number of positive results in this group of women, probably reflecting the higher sensitivity. This finding has to be taken into account when the AMPLICOR HPV test is used in the screening setting. As elegantly pointed out by Snijders et al. (24), it is necessary to distinguish between the clinical sensitivity and the analytical sensitivity. Whether a method with a higher analytical sensitivity would result in a better performance in terms of clinical sensitivity and specificity is still a matter of investigation. It could be suggested that, for epidemiological purposes, the highest sensitivity is better, while for clinical purposes, there might be a viral load threshold below which the HPV infection could be not relevant (10, 25, 26).

Another important measure of a diagnostic test is the rate of false-negative results. In our study, we had only one SCC patient who was correctly identified by both tests. The AMPLI COR HPV test detected all of the CIN3 patients (7 patients), while the HC2 assay failed to detect 2 of 7 patients. In these two CIN3 HC2 assay-negative patients, the genotyping demonstrated the presence of HPV genotype 16 alone in one

sample and HPV genotype 39 plus HPV genotype 45 in the other. We might speculate that these samples contained a low copy number of the HPV genome or that a deficient sample quality may have played a role. Unfortunately, it was not possible to determine the viral load, but similar experiences with false-negative results for HR HPV detection with HC2 have been reported previously (9, 13). In any case, the low number of samples does not allow us to draw any definite conclusion.

The slight differences observed between the two tests by the present investigation may have some clinical implications. At present, clinical use of HPV testing is directed to screening, triage of borderline smear results, and follow-up after conservative treatment of CIN. In the screening setting, the HPV test is generally used in combination with a Pap smear. The higher rate of positive results in patients with a normal smear observed for the AMPLICOR HPV test in comparison with the HC2 assay suggests that the latter is more suitable for the screening setting, where the management of cytology-negative and HR HPV-positive patients is still a clinical dilemma. Conversely, in the present study, the AMPLICOR test showed a slightly better accuracy in detecting CIN3 in comparison with the HC2 assay. While this is a minor advantage in the screening setting, where CIN3 is a rare occurrence and the HPV test is always coupled with a Pap smear, it may represent a true improvement in the triage of borderline smear results, where the rate of high-grade lesions is higher and the correct identification of the disease is of utmost importance for the subsequent management of these patients. In addition, it has recently been emphasized that, besides detecting the presence of one of the HR HPV genotypes using pooled probes, it could be of value to distinguish between the individual types and, in particular, to detect the presence of HPV 16 and/or HPV 18. These two HPV genotypes have been shown to be far more aggressive and dangerous than the other HR HPV genotypes, with 10-year cumulative incidences of ≥CIN3 among HPV genotype 16-positive and HPV genotype 18-positive women of 20% and 15%, respectively (10). Castle et al. reported that women with atypical squamous cells of undetermined significance or low-grade squamous intraepithelial lesions who were HPV genotype 16 positive had the highest 2-year risk for ≥CIN3 compared to women who were HPV negative; in particular, this risk was calculated to be fivefold greater than the increased risk in women who were positive for other oncogenic HPV types. The authors suggest that distinguishing the higher absolute risk for cervical precancer in HPV genotype 16-positive women from the lower risk posed by other oncogenic HPV types might have clinical implications (7). Their conclusions corroborate the fact that, besides the persistence of the infection, specific HPV types bear different oncogenic potentials. Therefore, if the future management of patients with preneoplastic lesions will include not only HR HPV detection but also HPV genotyping, the combination of any HPV assay with the LINEAR ARRAY will give adequate and appropriate information.

In conclusion, the present study shows that the two commercially available kits for HR HPV detection are easy to implement in the clinical laboratory, are reproducible, and give quite comparable results. The present data also evidence some slight differences in test performance, suggesting a different optimal clinical use, which should be further evaluated in further (prospective) clinical studies.

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