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Comparison of Two Commercial Assays for Detection of Human Papillomavirus (HPV) in Cervical Scrape Specimens: Validation of the Roche AMPLICOR HPV Test as a Means To Screen for HPV Genotypes Associated with a Higher Risk of Cervical Disorders

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Certain high-risk (HR) human papillomavirus (HPV) types are a necessary cause for the development of cervical disorders. Women with persistent HR HPV infections have an increased risk of developing high-grade cervical lesions, compared with those who have no or low-risk HPV infections. Therefore, implementation of HPV detection into cervical screening programs might identify women at risk of cervical cancer. Several HPV detection methods with different sensitivities and specificities are available. Recently, a new PCR-based technique, the Roche AMPLICOR HPV Test, was developed. This test recognizes a group of 13 HR HPV types simultaneously. This study was undertaken to validate and compare HPV detection in 573 cervical scrape specimens by the AMPLICOR HPV Test and the INNO-LiPA HPV detection/genotyping assay (SPF₁₀-LiPA system version 1). Human β -globin was not detected in nine specimens, which were therefore excluded from the comparison. Eleven scrape specimens containing HPV type 53 or 66 were also excluded from the comparison because these (probably) HR HPV types cannot be detected by the AMPLICOR HPV Test. The results of HPV detection by the Roche AMPLICOR HPV Test were confirmed by INNO-LiPA HPV detection/genotyping assay in 539/553 cases, showing an absolute agreement of 97.5% with a Cohen's kappa of 0.9327, indicating almost complete similarity of the two tests. Like the INNO-LiPA HPV detection/genotyping assay, the AMPLICOR HPV Test was sensitive, specific, feasible, and easy to handle. The value of the Roche AMPLICOR HPV Test with a broad-spectrum HR HPV detection has to be determined in prospective clinical studies.

Cancer of the uterine cervix is a major cause of death. Although screening programs to identify precursor lesions of cervical cancer have contributed to a reduction in mortality and morbidity due to this disease, 500,000 new cases of invasive cervical cancer are diagnosed annually and 230,000 women die because of this disease (29). In developing countries, cancer of the cervix is the most frequent female malignancy and is responsible for about 24% of all cancers in women. In developed countries, cervical cancer accounts for 7% of all female cancers (29).

The human papillomavirus (HPV) family consists of many different types; more than 100 types have been identified to date, of which 40 types have been detected in the anogenital mucosa (6, 11, 47). Several HPV types, such as types 16, 18, 31, 33, and 35, have been implicated as sexually transmitted agents with an etiological role in cervical carcinogenesis (27), whereas other types, such as types 6 and 11, are frequently detected in benign lesions such as condylomata acuminata. Therefore, HPV types 6 and 11 are termed low-risk (LR) HPV types, and

HPV types involved in carcinogenesis (such as types 16 and 18) are termed high-risk (HR) HPV types (27).

Infection by HR HPV types has been demonstrated in almost 100% of cervical carcinomas (42). Studies of the oncogenic potential of these HPV types have clearly demonstrated that HR HPVs are a necessary cause for the development of cervical cancer (4, 7, 17, 19, 34).

It is generally accepted that women infected with an HR HPV type are thus at higher risk of developing cervical cancer than those who are not infected with HPV or who are infected with one of the LR HPV types (10, 20, 30). Incorporation of HPV tests into screening programs might identify women who are at risk of developing invasive cervical cancer. Indeed, several studies have demonstrated the potential relevance of HPV testing in cervical cancer screening programs and the management of patients with slight abnormal cytology (2, 5, 8, 10, 34, 37, 42). Furthermore, the absence of HR HPV in cervical smears would permit less aggressive management of women with mild or equivocal cytological abnormalities because they are unlikely to progress (16, 26, 33). The importance of HPV in the genesis of cervical neoplasm suggest that diagnosis of a specific HPV infection would dictate a particular clinical outcome and hence be useful in patient management. Because of the large number of both HR and LR anogenital HPV types, which are all able to induce cervical disorders, the detection

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and typing of a broad spectrum of different HPV types are mandatory, certainly in the phase in which the definitive progression markers have yet to be established.

Since HPV cannot be cultured efficiently and the clinical performance of serological assays is poor, diagnosis of HPV infection is almost entirely based on molecular tools (14). Before the era of amplification technology, Southern blot, dot blot, filter in situ, and in situ hybridization assays using HPV type-specific probes were generally used to detect and genotype HPV (23, 24, 43). However, applicability was limited because of low sensitivity, low specificity, or both or because of complex execution of techniques and low potential for automated implementation, which is required for processing a large number of clinical samples in a population-based screening design. New amplification techniques, such as PCR, nucleic acid sequence-based amplification, and ligase chain reaction, and advances in old techniques, including liquid hybridization (e.g., hybrid capture) (8), have revolutionized the potential of molecular screening for HPV in the past decade.

General detection assays, with a broad spectrum of specificity for HPV, are now widely used for the detection of HPV in clinical cervical material (2, 12, 22, 31), although the Hybrid Capture II assay (Digene) is the only commercially available HPV screening test on the market. The Hybrid Capture II assay for HPV DNA detection is a liquid-based hybridization assay capable of detecting 13 HR HPV genotypes simultaneously (1). In 2003, a newly developed PCR-based technique, the Roche AMPLICOR HPV Test, was launched. This test is also capable of detecting 13 HR HPV types, with simultaneous assessment of the presence of the human β -globin gene as a positive control. This test could, in principle, be used for screening purposes. However, no data are currently available on the performance and value of the assay in a clinical setting.

The purpose of this study was to examine the utility of the AMPLICOR HPV Test by comparing results with those obtained using the INNO-LiPA HPV detection/genotyping assay. The INNO-LiPA HPV detection/genotyping assay is capable of detecting and genotyping 25 different HPV types simultaneously and has proved to be sensitive, specific, simple, and rapid in the assessment of HPV (14, 21). A total of 573 anonymous cervical samples were tested by both assays, and the results were compared.

MATERIALS AND METHODS

Cervical scrape specimens were obtained from 573 women seeing a gynecologist for routine cervical screening. Specimens were collected using the Cervexbrush (Roovers, Oss, The Netherlands) and processed using a liquid-based (Cytec) approach that provides monolayer distributions for cytological assessment and the possibility of isolating DNA for HPV detection assays. This method has received approval for clinical use from the U.S. Food and Drug Administration (18, 36). Cytological classification was performed by an experienced cytopathologist.

Specimen preparation. For isolation of nucleic acid from cervical scrape specimens in liquid cytology medium, the MagnaPure LC Isolation station (Roche Applied Science) was used; 200 μ l of material was isolated using the Total Nucleic Acid isolation kit (Roche Applied Science) as described by the manufacturer. Nucleic acid was resuspended in a final volume of 100 μ l; 10 μ l was used for PCR analysis.

After isolation of DNA, samples were tested for the presence of HPV by the INNO-LiPA HPV detection/genotyping assay and the Roche AMPLICOR HPV Test. All HPV tests were performed by investigators unaware of the cytological status or the results of the comparative HPV detection test.

INNO-LiPA HPV detection and genotyping. (i) PCR amplification of HPV

DNA. Broad-spectrum HPV DNA amplification was performed using a short PCR fragment assay (INNO-LiPA HPV detection/genotyping assay, SPF₁₀ system version 1, manufactured by Labo Biomedical Products bv, Rijswijk, The Netherlands). This assay amplifies a 65-bp fragment of the L1 open reading frame and allows detection of at least 43 different HPV types (15, 21). The SPF₁₀ PCR was performed with a final reaction volume of 50 μ l containing 10 μ l of the isolated DNA sample, 10 mmol/liter Tris-HCl (pH 9.0), 50 mmol/liter KCl, 2.0 mmol/liter MgCl₂, 0.1% Triton X-100, 0.01% gelatin, 200 μ mol/liter of each deoxynucleoside triphosphate, 15 pmol each of the forward and reverse primers tagged with biotin at the 5' end, and 1.5 U of AmpliTaq Gold (Perkin-Elmer). The mixture was incubated for 9 min at 94°C, 40 cycles of 45 s at 45°C, and 40 cycles of 45 s at 72°C, with a final extension of 5 min at 72°C. Each experiment was performed with separate positive and negative PCR controls.

The presence of HPV DNA was determined by hybridization of SPF₁₀ amplicons to a mixture of general HPV probes recognizing a broad range of HPV genotypes, in a microtiter plate format, as described previously (15).

(ii) HPV genotyping by reverse hybridization using the INNO-LiPA HPV genotyping system. A poly(dT) tail was enzymatically added to the 3' end of each of 25 oligonucleotides specific for 25 different types, namely, types 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68/73, 70, and 74. The tailed probes were applied as horizontal lines to membrane strips (manufactured by Labo Biomedical Products bv, Rijswijk, The Netherlands). The HPV genotyping assay was performed as described previously (14). Briefly, equal volumes (10 μ l each) of the biotinylated PCR products and denaturation solution (400 mmol/liter NaOH, 10 mmol/liter EDTA) were mixed in test troughs and incubated at room temperature for 5 min, after which 1 ml of prewarmed (37°C) hybridization solution (3 \times SSC [1 \times SSC is 0.15 mol/liter NaCl plus 0.015 mol/liter sodium citrate], 0.1% sodium dodecyl sulfate) was added, followed by the addition of one strip per trough. Hybridization was performed for 1 h at 50 \pm 0.5°C in a closed water bath with back-and-forth shaking. The strips were then washed twice with 1 ml of wash solution (3 \times SSC, 0.1% sodium dodecyl sulfate) at room temperature for 20 s and once at 50°C for 30 min. Following this stringent washing, strips were rinsed twice with 1 ml of a standard rinse solution (14). Strips were then incubated on a rotating platform with an alkaline phosphatase-labeled streptavidin conjugate diluted in a standard conjugate solution, at 20 to 25°C for 30 min, after which strips were washed twice with 1 ml of rinse solution and once with standard substrate buffer; color development was initiated by the addition of 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium to 1 ml of substrate buffer. After 30 min of incubation at room temperature, the color reaction was stopped by aspiration of the substrate buffer and addition of distilled water. After drying, the strips were visually interpreted using a grid.

AMPLICOR HPV Test. The AMPLICOR HPV Test utilizes amplification of target DNA by PCR followed by nucleic acid hybridization for the detection of 13 HR HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68).

PCR amplification of HPV DNA. The AMPLICOR HPV Test amplifies a sequence of nucleotides within the polymorphic L1 region of the HPV genome that is approximately 165 bp in length. A pool of 12 HPV primers present in the master mix (components comprise nucleotides, enzyme cofactor, Taq DNA polymerase, AmpErase enzyme [Applied Biosystems], buffers, and primers) is designed to amplify DNA from 13 HR HPV types. Capture probe sequences are located in polymorphic regions of L1 bound by these primers. An additional primer pair targets the human β -globin gene (268-bp amplicon) to provide a control for cell adequacy.

PCR was performed with a final reaction volume of 100 μ l containing 50 μ l AMPLICOR HPV master mix and 10 μ l of the isolated DNA. The mixture was incubated for 2 min at 50°C and 9 min at 95°C, followed by 40 cycles of 30 s at 95°C, 45 s at 54°C, and 30 s at 72°C, with a final extension at 72°C for at least 10 min to a maximum of 1 h.

Hybridization. After amplification, 100 μ l of AMPLICOR denaturation solution was added to each PCR tube, followed by incubation for 10 min at room temperature to allow complete denaturation. Two separate 96-microwell plates were used for detection of amplified HPV and β -globin. AMPLICOR hybridization buffer (100 μ l) was added to each well of both plates; 25 μ l of denatured amplified sample was then added to the appropriate wells of each detection plate. The plates were covered and incubated for 1 h at 37°C to allow the amplicons to bind to the oligonucleotide probes.

Detection. The plates were washed five times with AMPLICOR wash buffer using a BioTek MWP washer to remove unhybridized material. AMPLICOR avidin-horseradish peroxidase conjugate (100 μ l) was pipetted into the wells, and the plates were covered and incubated for 15 min at 37°C. The plates were again washed as described above. A 100- μ l aliquot of AMPLICOR substrate, containing tetramethylbenzidine and hydrogen peroxide, was added to each well, and plates were incubated in the dark at room temperature for 10 min to allow color

development. AMPLICOR stop reagent (100 μ l) was pipetted into the wells, and the optical density of the reaction mixture was measured at 450 nm in a BioTek MWP autoreader. An absorbance reading of greater than 0.2 was considered positive for the presence of HPV and β -globin.

Agreement was measured by absolute agreement, as well as Cohen's kappa statistics, a measure of the agreement between two methods that is in excess of that due to chance.

RESULTS

A total of 573 cervical samples were available for comparison. In 9/573 samples (1.5%), the human β -globin gene did not test positive by the AMPLICOR HPV Test, and these samples were therefore excluded from the comparison.

After HPV detection by both tests, INNO LiPA genotyping was performed in order to compare the two test results. HPV genotyping was performed on the positive samples. As the INNO LiPA HPV detection/genotyping assay detects HR HPV types, LR HPV types, and HPV X individually, in contrast to the AMPLICOR HPV Test, which detects only 13 HR HPV types as a group, the samples testing positive for LR HPV DNA or HPV X by the INNO LiPA HPV genotyping assay were considered negative in the comparison (55 samples). Of these 55 samples, 21 samples contained LR HPV DNA and 34 samples contained HPV X. In 51 of these 55 samples, the cytological result was normal whereas two samples were cytologically diagnosed as very mild dysplasia (later termed as atypical squamous cells of undetermined significance [ASCUS]), one scrape specimen was diagnosed as mild dysplasia, and for one scrape specimen no diagnosis could be assessed. In four patients HPV6 was detected, in one patient HPV42 was detected, in five patients HPV44 was detected, in three patients HPV54 was detected, in 1 patient HPV70 was detected, in three patients HPV74 was detected, and in four patients double infections with LR HPV types were detected.

Although HPV53 and HPV66 are considered probably carcinogenic (27), these genotypes are not included in the AMPLICOR HPV Test and were therefore excluded from the direct comparison although HPV53 was found by INNO-LiPA HPV genotyping in seven patients with a normal cytological result and HPV66 was found in three patients with a normal cytological result and in one patient with a normal smear result both types 53 and 66 were detected. So the final comparison was made for 553 cervical samples.

HR HPV DNA was detected by both the AMPLICOR HPV Test and the INNO LiPA HPV genotyping assay in 132/553 cervical samples (24%). A total of 407/553 cervical samples tested negative for HPV DNA in both tests (74%). Therefore, both tests showed corroborative results for HR HPV types in 539/553 samples (Table 1 and Table 2), demonstrating a concordance of 97.5% and $\kappa = 0.9327$ (confidence interval, 0.8980 to 0.9675).

The relationship between concordant HPV detection for both tests and cytological classification is shown in Table 2. HR HPV DNA was detected by both methods in 88/469 scrape specimens (19%) from women diagnosed with a normal cytological classification. HPV positivity increased with the severity of the cytological diagnosis: HR HPV DNA was detected in 63% (19/30) of women with ASCUS, 67% (18/27) of women with mild to moderate disorders, and 83% (5/6) of women with severe dysplasia. HPV DNA was detected in 29% (2/7) of

TABLE 1. Overview of the 553 samples tested by the AMPLICOR HPV Test and SPF₁₀-LiPA for HR HPV genotypes only^a

Test result	No. of samples		Total
	SPF ₁₀ -LiPA positive	SPF ₁₀ -LiPA negative	
AMPLICOR HPV positive	132	5	137
AMPLICOR HPV negative	9	407	416
Total	141	412	553

^a Absolute agreement, 97.5%; kappa = 0.9327 (confidence interval, 0.8980 to 0.9675).

women in whom cytological results could not be ascertained due to sampling errors. A single HR HPV infection was detected by the INNO-LiPA HPV detection/genotyping assay in 87/132 HPV-positive scrape specimens (66%). HPV types 16, 18, 31, 33, 51, and 52 accounted for 77% of the single HPV infections, with HPV type 16 being the most common (accounting for 34% of all single infections). In total, 45/132 positive cervical scrape specimens (34%) contained two or more HPV types (Table 1). Multiple (i.e., two or more) infections as a proportion of total infections were seen in all cytological classifications (normal, 38%; ASCUS, 17%; mild/moderate dysplasia, 19%; severe dysplasia, 17%; no diagnosis, 14%). The distribution of the individual HPV types within the multiple infections showed no statistically significant difference between the different cytological classifications, probably due to the unequal numbers in the groups. Since the AMPLICOR HPV Test only determines positivity, multiple infections could not be assessed by this method.

In 14 cases (2.5%), the HPV test results were discordant, as shown in Table 3. The AMPLICOR HPV Test detected HR HPV DNA in five cervical scrape specimens in which the INNO LiPA HPV genotyping assay was either negative (one case), positive for LR HPV (one case), or positive but where the type could not be determined (HPV X). In nine cases, the AMPLICOR test results were negative, whereas the SPF₁₀-LiPA detected HR HPV types in these nine cases. HPV18 and -31 were both missed in one case, HPV33 was missed twice, and HPV51 and HPV52 were missed in three cases by the AMPLICOR test. Twelve cervical scrape specimens in the discordant group were cytologically diagnosed as normal, one smear was classified as ASCUS, and the other as mild/moderate dysplasia.

DISCUSSION

Infections with HR HPV are now considered, beyond reasonable doubt, to be a necessary cause of the development of cervical cancer (3, 45). Women with persistent cervical HR HPV infections have a 100- to 300-fold increased risk of developing a high-grade cervical intraepithelial lesion (CIN 3) (13, 28, 30, 32, 42, 44). Although the sensitivity of HR HPV detection for identifying women with high-grade cervical intraepithelial neoplasia (CIN) or cervical cancer appears to be higher than that of conventional cytology, especially in women with borderline and mild dysplastic smear results, its clinical specificity is lower (1, 9, 33). Large randomized controlled trials are currently being conducted to investigate whether the addition of HR HPV detection to conventional cytology may

TABLE 2. Concordant HPV detection and typing in relation to the cytological classification for both PCR tests

Cytology result	No. of samples	% HR HPV positive	No. of samples with following HR HPV genotype:															
			16	18	31	33	35	39	45	51	52	56	58	59	68	Double	Triple	Multiple
Normal	469	19	18	4	4	2	1	4	2	4	7	3	2	3	1	26	5	2
ASCUS	30	63	5	2	1	1	1	1			3					3	1	1
Mild/moderate dysplasia	27	67	6	1	2		1			1	2					3	2	
Severe dysplasia	6	83	1			1				1	1					1		
No diagnosis	7	29					1									1		
Total	539	24																

improve the effectiveness of organized cervical cancer screening programs (5, 40). Identification of HR HPV types may identify women with an increased risk of cervical disease and may therefore provide additional clinical value (10). Indeed, in the United States, the American College of Obstetrics and Gynecology and the American Cancer Society both approved that women over 30 years old should be screened with an HPV DNA test combined with a cytological examination.

If HPV testing is to be introduced into cervical cancer prevention programs, then standardized, reliable, and accurate HPV tests are warranted (35). The newly developed, PCR-based AMPLICOR HPV Test may fulfill these demands. However, the test should be evaluated in a clinical laboratory setting. In this study, we compared HPV status in cervical scrape specimens using the AMPLICOR HPV Test and the INNO LiPA HPV detection/genotyping assay. Both systems appeared to be highly concordant, with a kappa of 0.9235, demonstrating highly similar outcomes.

In nine cases, no human β -globin was detected, suggesting an inadequacy of cellular material, probably due to sampling errors. In 14/553 cervical scrape specimens (2.1%), no agreement was found; of these, 5 were INNO LiPA negative/AMPLICOR positive and 9 samples were SPF₁₀-LiPA positive/AMPLICOR negative. HPV33 was missed twice, and HPV51 and HPV52 were missed in three cases by the AMPLICOR test. Although these HPV types were identified by both HPV tests in the majority of the positive cases (see Table 2), these three HPV genotypes need to be watched carefully as additional studies are performed in the field.

The sensitivity of HPV detection methods is based on the threshold value of the viral load/viral concentration. Amplification detection assays, such as PCR-based techniques, are highly sensitive (low threshold of viral load detection), in comparison with liquid hybridization tests (high threshold), for example, the Digene Hybrid Capture 2 test. Snijders et al. have defined “analytical” and “clinical” sensitivity and specificity in order to distinguish clinically irrelevant and clinically relevant HPV positivity rates (39). Analytical sensitivity is defined as the proportion of HPV-positive women who are correctly identified by a given test (but clinically unimportant), whereas clinical sensitivity identifies the proportion of women with disease (i.e., women with \geq CIN 3) who are correctly identified by a positive HPV test. However, it must be remembered that HPV infection indicates a risk of having or developing a cervical lesion and is not equivalent to a morphological disorder. Though the natural history of HPV infections is not fully understood, the viral load is probably low during the first phase of infection but may increase over time, in parallel with the de-

velopment of cytological disorders (38). Therefore, sensitive methods to diagnose HPV infection may be required in order to realize a maximum negative predictive value for the development of HPV-associated cervical carcinoma, particularly since most cervical smears are classified as normal or ASCUS. New insights in the viral load threshold, however, may direct the future management of HR HPV-positive women, since a high viral load seems to be indicative for viral persistence and disease development while a low viral load is associated with clearance of an infection and even regression of cervical lesions, as had been reviewed recently (39).

Most current HPV screening protocols identify the presence of one of a pool of HR HPV types but do not identify the individual genotype or determine whether repeated positive tests are due to the persistence of one particular type; women who are infected with any HR HPV type are treated in the same way. On the other hand, Wallin and coworkers have found a strong concordance between the type of HPV found in the baseline smear, before the development of cervical carcinoma, and the HPV type found in the biopsy specimen of the invasive cancer (44), indicating that neoplasia is associated with HPV type-specific persistence. Therefore, future management of patients with neoplasia will probably include not only HPV detection but HPV genotyping as well.

It should also be considered that both the Hybrid Capture II assay and the AMPLICOR HPV Test do not detect HPV26, -53, -66, -73, and -82, HPV types that have been classified as probably HR (26, 53, 66) or HR (73, 82) types in a large epidemiological study of the oncogenic potential of HPV ge-

TABLE 3. Overview of discordant cases

SPF ₁₀ LiPA test result	AMPLICOR HPV test result
HPV X	Positive
HPV11, -18, -39, -68.....	Negative
HPV33.....	Negative ^a
HPV6.....	Positive
HPV31, -70	Negative
Negative	Positive
HPV33.....	Negative ^b
HPV X	Positive
HPV11, -42, -51, -52, -53	Negative
HPV52, -74.....	Negative
HPV51.....	Negative
HPV52.....	Negative
HPV51.....	Negative
HPV X	Positive

^a Cytological diagnosis: ASCUS.

^b Cytological diagnosis: mild/moderate dysplasia.

notypes (27). In our comparison, we would have missed 11 of 564 (1.9%) infections in women who were found positive for either HPV53 or HPV66 by the SPF₁₀-LiPA test. Obviously, as indicated above, these women would not all have developed cervical lesions or cancer, but Munoz et al. found 1.1% of the cervical cancers positive for either one of these five types (27).

It is clear that the application of HPV testing in cervical screening programs will require further improvements in, and standardization of, testing methods (35). At present, consensus guidelines on primary screening and/or the triage of women with mild cytological disorders are lacking because of conflicting data in the existing literature about the prevalence of HPV in women with normal, mild, or equivocal cytological abnormalities. The estimated point prevalence of genital HPV infections among populations of women with normal cytological smears ranges from 1.5% to 55% (41, 46) and shows an age-related pattern (25). These differences in the prevalence of HPV are dependent not only on the screened population but also on the clinical sample size, menstrual cycle, viral load, and detection method used for HPV assessment. Incorporation of HPV detection and typing into primary screening and/or triage of cytological disorders warrants consensus guidelines and the use of highly concordant HPV detection methods. Whether the AMPLICOR HPV Test fulfills these demands should be further evaluated in further (prospective) clinical studies.

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