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Evaluation of the SPF_{10}-INNO LiPA Human Papillomavirus (HPV) Genotyping Test and the Roche Linear Array HPV Genotyping Test

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The need for accurate genotyping of human papillomavirus (HPV) infections is becoming increasingly important, since (i) the oncogenic potential among the high-risk HPV genotypes varies in the pathogenesis of cervical cancer, (ii) monitoring multivalent HPV vaccines is essential to investigate the efficiency of the vaccines, and (iii) genotyping is crucial in epidemiologic studies evaluating HPV infections worldwide. Various genotyping assays have been developed to meet this demand. Comparison of different studies that use various HPV genotyping tests is possible only after a performance assessment of the different assays. In the present study, the SPF_{10} LiPA version 1 and the recently launched Roche Linear Array HPV genotyping assays are compared. A total of 573 liquid-based cytology samples were tested for the presence of HPV by a DNA enzyme immunoassay; 210 were found to be positive for HPV DNA and were evaluated using both genotyping assays (163 with normal cytology, 22 with atypical squamous cells of undetermined significance, 20 with mild/moderate dysplasia, and 5 with severe dysplasia). Comparison analysis was limited to the HPV genotype probes common to both assays. Of the 160 samples used for comparison analysis, 129 (80.6%) showed absolute agreement between the assays (concordant), 18 (11.2%) showed correspondence for some but not all genotypes detected on both strips (compatible), and the remaining 13 (8.2%) samples did not show any similarity between the tests (discordant). The overall intertest comparison agreement for all individually detectable genotypes was considered very good (κ value, 0.79). The genotyping assays were therefore highly comparable and reproducible.

Molecular and epidemiologic studies have shown that a persistent infection with high-risk human papillomavirus (HPV) is the most important risk factor for both cervical cancer and its precursors (9, 11, 29, 33). Approximately 40 different HPV types can infect the mucosa of the anogenital tract. Based on their carcinogenicities, these anogenital HPV types have been subdivided into low-risk HPV (lr-HPV) types, probable high-risk HPV (hr-HPV) types, and hr-HPV types (27), although some controversy remains regarding the probable high-risk genotypes (30). Almost all squamous cell cervical cancers worldwide harbor hr-HPV types (36). Moreover, high-risk HPV DNA can be detected in 74% of the premalignant low-grade cervical intraepithelial neoplasia (CIN) lesions and approximately 84% of the high-grade CIN lesions (25). Consequently, the efficacy of population-based screening programs solely using cervical cytology could benefit from adding hr-HPV testing (32). Accordingly, many ongoing international research projects assess the feasibility of introducing hr-HPV tests in available routine screening.

For these screening purposes, several tests have been developed in order to distinguish high-risk HPV infections from no HPV infection. Among these are the signal amplification method Hybrid Capture II (hc2) (Digene Corp., Gaithersburg, Maryland) and the recently developed target amplification method Roche AMPLICOR HPV test (Roche Molecular Systems, Inc., Branchburg, NJ) (35). Although both tests are commercially available and Conformité Européenne (CE) marked, hc2 is currently the only FDA-registered HPV screening assay (7). Both tests differentiate between an infection with one or more of 13 hr-HPV genotypes (genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) and no hr-HPV infection—an "hr-HPV plus/minus" screening. Although these tests are not designed to detect the recently described probable hr-HPV or any lr-HPV infection, some cross-reactivity outside of the spectrum of 13 hr-HPV genotypes has been reported for the hc2 assay (5). Neither the hc2 nor the AMPLICOR HPV assay allows the identification of specific genotypes (26), nor do they have the ability to identify infections involving multiple genotypes.

However, recent studies have provided evidence for a difference in oncogenic potential between the different hr-HPVs (6), arguing for the importance of HPV genotyping in addition to the "hr-HPV plus/minus" screening. Outside of the clinical setting, HPV genotyping is a key characteristic of studies evaluating the epidemiology of HPV infections worldwide. Although a number of HPV genotyping assays have been used in such studies, a reliable comparison between the diagnostic and epidemiological data generated is difficult, since data on the intertest comparisons between the different genotyping assays are limited.

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The SPF_{10}-INNO LiPA assay is capable of amplifying up to 43 different genotypes and providing type-specific genotype information for 25 different HPV genotypes simultaneously, has been extensively tested, and has proven to be highly sensitive and specific (15, 25). The Roche Linear Array (LA) HPV genotyping test (Roche Molecular Systems, Inc., Branchburg, NJ) is a recently launched new HPV genotyping assay able to genotype 37 HPV types, concurrently assessing human β-globin. The full spectrum of HPV genotypes amplified by the PGMY primer system (13) used in the Roche Linear Array HPV genotyping test has not been assessed beyond the 37 genotypes probed. In essence, both assays could be used for genotyping analysis.

This study was designed to compare these two well-known and commonly used commercially available genotyping assays with HPV DNA-positive samples.

MATERIALS AND METHODS

Cervical scrapes were obtained from 573 women attending the Department of Gynaecology for routine cervical screening. Specimens were collected using the Cervex-Brush (Rovers Medical Devices B.V., Oss, The Netherlands) and processed using a liquid-based cytology medium (ThinPrep; Cytyc Corp., Marlborough, MA) that provides monolayer distribution for cytological assessment. Moreover, it offers the opportunity to isolate DNA for various HPV detection assays. This method has received U.S. FDA approval for clinical use (20, 31).

Specimen preparation. For isolation of DNA from cervical scrapes in liquid-based cytology medium, the MagNAPure LC isolation station (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) was used; 200 μl of material was isolated using the MagNA Pure LC Total Nucleic Acid Isolation kit (Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim Germany), as described by the manufacturer. With each set of 28 cervical-scrape samples, four negative controls (distilled water) were used to monitor the DNA isolation procedure and to assess contamination. Nucleic acid was resuspended in a final volume of 50 μl; 10 μl was used for each of the various PCR analyses.

SPF_{10}-INNO LiPA HPV detection and genotyping (DNA enzyme immunoassay [DEIA] and LiPA). (i) PCR amplification of HPV DNA. Broad-spectrum HPV DNA amplification was performed using a short-PCR-fragment assay (SPF_{10} HPV PCR; Labo Bio-Medical Products B.V., 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 (16, 25). The SPF_{10} PCR system was used in a final reaction volume of 50 μl containing 10 μl of the isolated DNA sample and 40 μl of the PCR mixture, which contained 10 mmol/liter Tris-HCl (pH 9.0), 50 mmol/liter KCl, 2.0 mmol/liter $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin, 200 μmol/liter of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 15 pmol each of the forward and reverse primers tagged with biotin at the 5′ end, and 1.5 units of AmpliTaq Gold (Applied Biosystems, Foster City, CA). Activation of AmpliTaq Gold for 9 min at 94°C, was followed by 40 cycles of 30 s at 94°C, 45 s at 52°C, and 45 s at 72°C, with a final extension of 5 min at 72°C. Appropriate negative and positive controls were used to monitor the performance of the PCR method in each experiment.

(ii) HPV detection by DEIA. The presence of HPV DNA was determined by hybridization of SPF_{10} amplimers to a mixture of general HPV probes recognizing a broad range of high-risk, low-risk, and possible high-risk HPV genotypes in a microtiter plate format, as described previously (15, 25). All HPV DNA-positive samples (by SPF_{10} DEIA) were genotyped using the INNO-LiPA HPV genotyping assays and the Roche Linear Array HPV genotyping test as described below. Twenty randomly selected DEIA-negative samples that had previously tested negative by the Roche AMPLICOR HPV test (35) were also assessed using both genotyping assays.

(iii) HPV genotyping by reverse hybridization using the INNO-LiPA HPV genotyping system. The 28 oligonucleotide probes that recognize 25 different types (Table 1) were tailed with poly(dT) and immobilized as parallel lines to membrane strips (Labo Bio-Medical Products B.V., Rijswijk, The Netherlands). The HPV genotyping assay was performed as described previously (15). The LiPA strips were manually interpreted using the reference guide provided.

The samples that tested positive using the DNA enzyme immunoassay but that showed no results on the LiPA strip were considered to be HPV X type, i.e., genotypes not available on the LiPA strip.

TABLE 1. Distribution of HPV genotypes in the LiPA and LA assays

Oncogenic potential (26)	HPV genotype	Detection in[a]:	
		SPF_{10}-LiPA	LA
High risk	16	X	X
	18	X	X
	31	X	X
	33	X	X
	35	X	X
	39	X	X
	45	X	X
	51	X	X
	52	X	X
	56	X	X
	58	X	X
	59	X	X
	68	X[b]	X
	73	X[b]	X
	82		X
Probable high risk	26		X
	53[c]	X	X
	66	X	X
Low risk	6	X	X
	11	X	X
	34	X	
	40	X	X
	42	X	X
	43	X	
	44	X	
	54	X	X
	55		X
	61		X
	62		X
	64		X
	67		X
	69		X
	70	X	X
	71		X
	72		X
	74	X	
	81		X
	83		X
	84		X
	IS39		X
	CP6108		X

[a] X, detected.
[b] LiPA does not distinguish between HPV 68 and HPV 73, since both types are detected by a single probe.
[c] The oncogenic potential of HPV 53 is controversial (30).

Linear Array HPV genotyping test. The LA HPV genotyping test (Roche Molecular Systems, Inc., Branchburg, NJ) is a new qualitative in vitro test for the determination of 37 anogenital HPV DNA genotypes (Table 1). The LA test was applied to all samples that tested positive for HPV by DEIA and to 20 randomly selected DEIA-negative samples.

(i) PCR amplification of HPV DNA. The LA test uses biotinylated PGMY primers to amplify a 450-bp fragment within the polymorphic L1 region of the HPV genome. The PGMY amplification system has been described previously (13). The PGMY primers are present in the "master mixture" (containing buffer, nucleotides [dATP, dCTP, dGTP, and dUTP], $MgCl_2$, and <0.02% AmpliTaq Gold DNA polymerase) and amplify HPV DNA from 37 HPV genotypes, including 13 high-risk types (Table 1). Amplicons incorporate dUTP, allowing the use of AmpErase enzyme (uracil *N*-glycosylase), which is included in the master mixture to prevent PCR carryover contamination. 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, extraction, and amplification.

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TABLE 2. Distribution of 40 excluded samples that either showed only assay-unique genotypes or were HPV DNA positive but genotype negative (i.e., LiPA X type)

LA result	No. of samples with indicated result by SPF_{10}-LiPA		Total
	LiPA X type	Assay-unique genotype	
Negative	9	7	16
Assay-unique genotype	24	0	24
Total	33	7	40

TABLE 3. Overview of the 170 included samples with assay-common genotypes

Assay-unique genotype	No. of samples			Total
	Concordant	Compatible	Discordant	
None	87	24	21	132
LiPA	3	0	0	3
LA	20	12	1	33
LiPA and LA	2	0	0	2
Total	112	36	22	170

PCR was performed in a final reaction volume of 100 μl, containing 50 μl HPV master mixture, 40 μl PCR water, and 10 μl isolated DNA. The mixture was incubated for 2 min at 50°C and for 9 min at 95°C, followed by 40 cycles of 30 seconds at 95°C, 1 min at 55°C, and 1 min at 72°C, with a final extension at 72°C lasting from 10 min to a maximum of 1 h. The provided HPV-positive and -negative controls were used with each set of 10 samples to assess the performance of the reaction.

(ii) Hybridization and detection. Following amplification, the HPV and human β-globin amplicons were denatured by immediately adding 100 μl denaturation solution to each PCR tube. Hybridization and HPV genotyping were performed as described by the manufacture (Roche Molecular Systems, Inc., Branchburg, NJ). The strips were manually interpreted using the Linear Array HPV reference guide, by reading the individual types down the length of the strip. Samples that were both SPF_{10} DEIA and LA β-globin positive yet were not reactive to any of the genotype probes on the LA strip were considered "LA negative."

Design of the study. Previously, the samples had been assessed in an analysis comparing only high-risk HPV types detected by the Roche AMPLICOR HPV test and the INNO-LiPA HPV detection and genotyping assay (35). Since the present study compares two genotyping assays, only the DEIA HPV-positive samples and 20 randomly selected DEIA (and Roche AMPLICOR) HPV-negative samples were assessed. In order to have the most accurate comparison between the two genotyping tests, only the HPV genotypes identified by both assays (i.e., lr-HPV 6, 11, 40, 42, 54, and 70; possible hr-HPV 53 and 66; and hr-HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) were considered for direct comparison of the individual HPV genotypes (Table 1). These will be referred to as assay-common genotypes. High-risk HPV genotypes 68 and 73 were not taken into account for individual comparison, since these types are identified by a single probe in the LiPA assay and thus cannot be distinguished. Moreover, the classification of HPV 53 as possibly high risk is currently disputed. When comparing the two genotyping assays, results were termed concordant, compatible, or discordant based on the following definitions. If the analyses yielded identical assay-common genotypes in both tests, the results were termed concordant. Results were termed compatible if one or more additional assay-common genotypes were not detected by either of the assays. Genotyping results were termed discordant if there were no similarities in the assay-common genotypes between the two tests. Assay results for HPV genotypes uniquely identified by each of the two assays (i.e., assay-unique HPV genotypes 34, 43, 44, and 74 detected only by LiPA and the assay-unique HPV genotypes 26, 55, 61, 62, 64, 67, 69, 71, 72, 81, 82, 83, 84, IS39, and CP6108 detected solely by the LA test) were not considered in determining concordant, compatible, or discordant status.

From all compatible and discordant samples, a reextracted DNA sample was randomly retested in a blind approach in a discrepancy analysis using both genotyping assays. Eleven concordant samples (six single infections, four double infections, and one triple infection) and six double-negative (i.e., DEIA-positive, LiPA X-type, and LA-negative) samples were used as positive and negative controls for both inter- and intra-assay performance control.

All HPV tests were performed by investigators unaware of the results of the comparative HPV detection or genotyping tests.

Statistics. All data were analyzed using SPSS version 12.0.1. for Windows. Agreement was measured by absolute agreement and Cohen's kappa statistics, a measure of the agreement between two methods that is in excess of that due to chance.

RESULTS

In total, 218 of the 573 DNA samples tested positive by SPF_{10} DEIA. These were considered suitable for analysis using the SPF_{10} LiPA and LA HPV genotyping assays. Eight samples were excluded from further analysis: four showed negative β-globin results in the LA test, and for four other samples, insufficient material was available to perform adequate assessments. Twenty randomly selected DEIA-negative control samples were negative in both genotyping assays and were thus not taken into consideration for further analysis. Of the 210 DEIA-positive samples, 163 (77.6%) indicated normal cytology. Atypical squamous cells of undetermined significance (ASCUS) were detected in 22 samples (10.5%), mild/moderate dysplasia was observed in 20 samples (9.5%), and 5 samples (2.4%) showed severe dysplasia.

Of the 210 DEIA-positive samples tested using both genotyping assays, 40 samples were excluded, since one of the tests was negative whereas the comparative test detected an assay-unique genotype or LA was negative and LiPA showed an X type (Table 2).

In 132 of the remaining 170 samples, all detected genotypes could have been identified by both assays. Of the samples harboring only assay-common genotypes, 87/132 (65.9%) were concordant, 24 (18.2%) were compatible, and 21 (15.9%) showed discordant results (Table 3). Finally, in 38 cases, assay-unique genotypes were detected in addition to assay-common genotypes. Of these samples, 25 (65.8%) had concordant results, 12 (31.6%) were compatible, and one (2.6%) was discordant. In the final analysis of 170 samples, these 38 samples were retained. The additional assay-unique genotypes found in these 38 samples were not taken into consideration. The outcomes of the concordant, compatible, and discordant cases are described in detail below.

Concordant cases. Of the 112 concordant cases (25 with and 87 without assay-unique genotypes), 69 (61.6%) contained a single HPV genotype and the remaining 43 samples contained multiple genotypes. Thirty-two samples (28.6%) harbored two different genotypes, eight samples (7.1%) contained three HPV genotypes, and three samples (2.7%) contained four genotypes. One or more high-risk genotypes were detected in 86.6% (97/112) of these samples, whereas seven samples (6.3%) contained only low-risk genotypes and eight samples (7.1%) also harbored probable hr-HPV genotypes.

Compatible cases. All 36 compatible cases were multiple infections. The LiPA assay did not detect a total of 41 genotypes in 30 separate clinical samples. In 23 cases, 1 type was missed; in 5 cases, 2 types were missed; and in 2 cases, 4 types

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TABLE 4. Overview of the 36 compatible and 22 discordant samples

Oncogenic potential	Genotype	No. of specific genotypes not detected			
		Compatible samples		Discordant samples	
		LiPA	LA	LiPA	LA
High risk	16	7			1
	18	2	1		
	31	2	2		
	33	1			2
	35				1
	39	3			
	45	2		1	
	51		1		4
	52	1	1		2
	56		3	1	1
	58	3			
	59	5			
	68/73	1	1		2
Probable hr	53		1	1	3
	66	1			2
Low risk	6				2
	11	1	2		
	42	4		1	
	54	8		2	1
Total		41	12	6	21

were missed (13 low-risk, 1 possible high-risk, and 27 high-risk genotypes were not detected by the LiPA test). The Linear Array assay, on the other hand, did not detect 12 genotypes in eight separate samples. In six cases, 1 type was missed; in one case, 2 types were missed; and in one case, 4 types were missed (2 low-risk, 1 possible high-risk, and 9 high-risk HPV types). Table 4 gives an overview of the individual types that were not detected. Fifteen of the 16 cases in which LiPA missed an hr-HPV type were samples infected with multiple hr-HPV types that tested positive for another high-risk type, which was also detected in the LA.

Discordant samples. In 22 (12.9%) of the 170 samples considered, no similarity was observed between the genotypes found in the two tests. These were predominantly single infections. An overview of the individual discordant cases is given in Table 4. Twenty-seven genotypes were discrepant between the two assays in 22 different samples. The LA test did not detect 13 hr-HPV, 5 probable hr-HPV, and 3 lr-HPV types that were found to be positive in the LiPA assay. The LiPA assay, on the other hand, failed to detect two high-risk, one probable high-risk, and three low-risk types, which were all found to be positive on the LA strip.

The genotypes that were detectable by both assays among all 170 samples (112 concordant, 36 compatible, and 22 discordant) were individually compared, as summarized in Table 5. The overall strength of agreement between the two assays for the individual genotypes was considered good ($\kappa = 0.792$). Although HPV 16 was detected in 45 samples using the LA test and in 39 samples using the LiPA, agreement between the tests was considered very good, with a κ value of 0.874. The agreement between the two assays for the other high-risk and probable high-risk genotypes varied between "good" and "very good." The agreement between the two tests for the low-risk genotypes was "moderate" to "perfect." The agreement for

TABLE 5. Kappa values and *P* values by McNemar's test for individual HPV genotypes detectable by both assays[a]

Oncogenic potential	Genotype	No. of genotypes found positive by:			κ value (95% CI)[b]	*P* value (McNemar's test)
		LiPA	LA	LiPA and LA		
High risk	16	39	45	38	0.874 (0.788–0.959)[d]	0.08
	18	14	15	13	0.887 (0.760–1.014)[d]	1.00
	31	13	13	11	0.833 (0.672–0.995)[d]	0.62
	33	10	9	8	0.833 (0.645–1.020)[d]	1.00
	35	9	8	8	0.938 (0.817–1.059)[d]	1.00
	39	7	9	7	0.869 (0.687–1.050)[d]	0.48
	45	5	8	5	0.761 (0.492–1.029)[e]	0.25
	51	16	11	11	0.799 (0.626–0.973)[e]	0.07
	52	23	21	20	0.896 (0.795–0.997)[d]	0.62
	56	12	9	8	0.747 (0.528–0.965)[e]	0.37
	58	8	11	8	0.833 (0.646–1.020)[d]	0.25
	59	6	11	6	0.692 (0.426–0.958)[e]	0.07
Probable hr	53	20	17	16	0.848 (0.718–0.979)[d]	0.37
	66	9	8	7	0.814 (0.606–1.023)[d]	1.00
Low risk	6	11	9	9	0.894 (0.748–1.040)[d]	0.48
	11	4	3	2	0.563 (0.072–1.053)[f]	1.00
	40	0	0	0		
	42	2	7	2	0.434 (−0.055–0.923)[f]	0.07
	54	9	18	8	0.562 (0.311–0.812)[f]	0.02
	70	6	6	6	1.000 (1.000–1.000)[c]	

[a] The results for 112 concordant, 36 compatible, and 22 discordant samples after initial analysis are shown.
[b] CI, confidence interval.
[c] Strength of agreement considered perfect.
[d] Strength of agreement considered very good.
[e] Strength of agreement considered good.
[f] Strength of agreement considered moderate.

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TABLE 6. All genotyping and comparison results for the 35 initially compatible and discordant samples assessed by discrepancy analysis

Initial comparison	HPV genotype(s) (initial analysis)		Discrepancy comparison	HPV genotype(s) (discrepancy analysis)	
	LiPA_1	LA_1		LiPA_2	LA_2
Compatible	35	33, 35	Concordant	33, 35	33, 35
Compatible	51	16, 39, 51	Concordant	51	51
Compatible	18, 33	18, 31, 33	Concordant	18, 33	18, 33
Compatible	33	16, 33	Concordant	33	33
Compatible	68/73	58, 73	Concordant	68/73	73
Compatible	39	16, 39	Concordant	39	39
Compatible	52, 53	52, 53, 54, 67	Concordant	52, 53, 54	52, 53, 54, 67
Compatible	35, 39, 70	16, 35, 39, 70, 81	Concordant	35, 39, 70	35, 39, 70, 84
Compatible	16	16, 59	Compatible	16	16, 59
Compatible	6, 51	6, 16, 18, 39, 51, 66	Compatible	6	6, 16, 18, 39, 51, 66
Compatible	51, 52, 53, 59	45, 51, 52, 53, 59, IS39	Compatible	53	42, 51, 52, 53, 59, IS39
Compatible	6, 33	6, 33, 58, 59, 72	Compatible	6, 31, 33, 58, 59	6, 33, 58, 59, 72
Compatible	6, 16, 52	6, 16, 42, 52	Compatible	6, 16, 52	6, 16, 42, 52
Compatible	52	16, 52	Compatible	6, 16, 52, 56	16, 52
Compatible	6	6, 59	Compatible	6	6, 59
Compatible	31, 70	31, 54, 62, 70	Compatible	6, 31, 70	62, 70
Compatible	54	54, 73	Compatible	54	54, 73
Compatible	16	11, 16, 59, 81	Compatible	16	11, 16, 59, 81
Compatible	56, 66, 68/73	39, 52, 56, 66, 68	Compatible	56, 66, 68/73	52, 56, 66, 68
Compatible	16, 52	16, 52, 54	Compatible	16, 52	16
Compatible	53	42, 53, IS39	Compatible	53	42, 51, 53, 59, IS39
Compatible	53, 66	16, 53, 66	Compatible	66	53, 66
Compatible	31, 33, 53	33, 42, 45, 53, 54, 59, 61, 83	Compatible	31, 33, 45, 53, 59	33, 42, 45, 53, 54, 59, 61, 83
Compatible	56, 58	54, 56, 58, 62	Compatible	56, 58	54, 56, 58, 62
Compatible	54, 56	54	Compatible	54, 56	54
Compatible	16, 31, 53, 58	16, 18, 53, 54, 62, CP6108	Compatible	16, 18, 31, 53, 58	16, 53, 54, 58, 62, CP6108
Compatible	33	33, 54	Discordant	33	54
Compatible	56, 66	66, 67	Discordant	56, 66	67
Compatible	56, 59	59	Discordant	X type	59
Compatible	51, 53	51, 53, 54, 62	Discordant	51, 53	62
Discordant	6	Negative	Concordant	6	6
Discordant	6, 53	Negative	Concordant	6, 53	6, 53
Discordant	X-type	53	Concordant	X type	Negative
Discordant	16	Negative	Concordant	16	16
Discordant	X-type	45, 61, 83	Concordant	45	45, 61, 83
Discordant	53	Negative	Concordant	X type	Negative
Discordant	52	Negative	Concordant	X type	Negative
Discordant	53	Negative	Concordant	X type	Negative
Discordant	52	54	Concordant	X type	Negative
Discordant	66	Negative	Discordant	66	68
Discordant	35	Negative	Discordant	35	Negative
Discordant	56	Negative	Discordant	56	Negative
Discordant	X-type	42	Discordant	X type	42
Discordant	68/73	Negative	Discordant	68/73	Negative
Discordant	51, 66, 68/73	Negative	Discordant	51	Negative
Discordant	X-type	56	Discordant	X type	56
Discordant	51	Negative	Discordant	51	Negative
Discordant	51	Negative	Discordant	51	Negative

HPV 54 was moderate, since LiPA and LA shared 8 samples harboring the low-risk genotype whereas LA detected it in 10 additional samples. Also, the agreement for lr-HPVs 11 and 42 was moderate, while HPV 70 was detected in equal amounts by both assays. Low-risk HPV 40 was not detected in either of the tests; thus, no agreement could be calculated. The difference in detection of lr-HPV 54 was statistically significant ($P < 0.05$; McNemar's test). Although the differences for hr-HPV 16, 51, and 59 and lr-HPV 42 between the assays were large, they were considered not quite statistically significant ($P > 0.07$; McNemar's test). In the individual comparison of the other genotypes, no statistically significant differences were detected.

Discrepancy analysis. The compatible ($n = 36$) and discordant ($n = 22$) samples were reanalyzed using the two genotyping assays in a discrepancy analysis. DNA was reextracted from these 58 compatible/discordant samples. As interassay test controls, 11 previously concordant (6 single and 5 multiple infections) and 6 previously double-negative samples (LiPA X type and LA negative) were also included; these samples were used for method performance assessment only. All 6 double-negative samples remained negative, and all 11 concordant samples appeared identical in both second genotyping assays. These internal controls were not further considered in the discrepancy analysis. Of the 58 discrepant samples, 10 were β-globin negative by the Linear Array and were also negative by LiPA. Of these 10 samples, 6 had been concordant and 4 had been discordant; these 10 samples were excluded from the discrepancy analysis. The crude initial and discrepancy analysis

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TABLE 7. Intra-assay comparison overview of the 65 samples reanalyzed in the discrepancy analysis, including the 17 control samples concordant in all four assays

Tests compared[a]	No. of samples			Total
	Concordant	Compatible	Discordant	
1st LiPA vs 2nd LiPA	48	11	6	65
1st LA vs 2nd LA	43	16	6	65

[a] 1st, initial comparison; 2nd, discrepancy comparison.

results for the remaining 48 samples are shown in Table 6. Of the 30 compatible samples from the initial analysis, 18 remained compatible after discrepancy analysis, while 8 appeared concordant and 4 discordant in a comparison of the second genotyping assays. Of the 18 discordant samples from the first test run, 9 remained discordant in the second analyses between LiPA and LA, whereas 4 appeared genotype concordant and 5 were concordant as LiPA X type, LA negative. Thus, comparing the second LiPA and LA tests yielded 17 concordant, 18 compatible, and 13 discordant results.

Intra-assay comparisons taking these 48 samples and the 17 control samples in both initial and discrepancy analyses into account showed highly comparable results for the two assays (Table 7).

In conclusion, of the 160 samples considered for final analysis, 80.6% (129/160) showed identical results, 11.2% (18/160) appeared compatible, and 13 samples (8.2%) were discordant.

DISCUSSION

Based on this study, we can conclude that the SPF_{10}-INNO LiPA and the Linear Array HPV genotyping assays are highly congruent for the genotypes detectable in both assays. Moreover, the manageabilities of both the SPF_{10}-INNO LiPA and the Linear Array assays are highly comparable, as are to a large extent the total run times required for both assays, including for amplification and preparation of all of the reagents.

Generally, a separate screening is needed preceding genotyping in order to assess a sample's HPV DNA positivity, i.e., an HPV plus/minus screening. An advantage of the LiPA is the use of the same amplicon for both detection of 43 different lr-, probable hr-, and hr-HPV genotypes and genotyping of 25 different HPVs. For the LA, a prescreening test with the PGMY primers is available using a generic HPV probe labeled with digoxigenin in a microtiter plate-based assay, as recently described (18). Without the need for further amplification, this amplicon can be directly used for the Linear Array genotyping assay. However, the efficiency of such a combination has not been studied. The recently launched HPV Roche AMPLICOR test for HPV plus/minus screening is not meant for an LA screen. It could also be used as a pretest, but the assay detects only high-risk HPV types (35).

In the initial comparison, i.e., prior to the discrepancy analysis, LiPA did not detect 27 high-risk genotypes in 30 compatible cases. Evidently, all the cases involved were multiple infections, i.e., containing two or more HPV types. Apparently, if an infection encompasses multiple genotypes, the SPF_{10}-INNO LiPA assay is less sensitive than the LA. After finding analogous results using the LiPA assay, Van Doorn et al. propounded the idea of PCR competition between genotypes in mixed infections and suggested a combined testing algorithm using broad-spectrum and type-specific PCRs for HPV 16 and HPV 18 (L. J. van Doorn, A. C. Molijn, B. Kleter, W. G. V. Quint, and B. Colau, Abstr. 22nd IPV Conf., abstr. N-01, 2005). The complexity of assessing multiple genotypes was addressed previously (34). Amplification and identification of two genotypes present in equimolar amounts are likely possible. However, "primer competition" between genotypes might occur if one genotype is present in molar excess, outcompeting the other (34). In the present study, this was demonstrated by the samples harboring multiple infections that were not identically genotyped by both assays. Also, LA detected hr-HPV 16 in seven samples that were LiPA HPV 16 negative; after the second LA, however, five samples no longer showed HPV 16. Moreover, in a previous study Van Doorn and colleagues detected HPV 16 and HPV 18 using type-specific PCR in samples negative for these genotypes (but not for other genotypes) using general primer sets (34). In the present study we observed similar results (data not shown). Although the viral load was not determined in the present study, low-copy-number samples have previously shown more discrepancies in intralaboratory and interlaboratory comparisons (17).

The LA assay is unable to distinguish hr-HPV 52 from other high-risk genotypes (33, 35, and 58). This could be inconvenient in future studies using the Linear Array, since hr-HPV 52 is prevalent in approximately 5% of the HPV-positive women with normal cytology (8) and causes 2.2% of all cervical cancers (27). In 19 samples from the present study, hr-HPV 52 positivity could not be excluded based on LA genotyping. However, in these cases, the comparative LiPA tests did not detect this specific genotype. Two samples were considered Linear Array HPV 52 positive based on the LiPA results.

Among the 22 discordant cases, the number of hr-HPV genotypes detected by the Linear Array was not higher than the number detected by LiPA. All but three of these samples were single infections, predominately HPV 33, HPV 51, and HPV 52. A higher inclusivity level has been observed for some high- and low-risk HPV genotypes, particularly hr-HPV 33 and hr-HPV 56, when the PGMY amplification system is used (see the product insert for the CE-marked Linear Array HPV genotyping test, European market). The inclusivity level equates to the lowest concentration (copies/ml) that shows a 100% positive hit rate in a replicate of six tests or the concentration that is the probit-predicted 95% positive hit rate. This could explain some of the differences between the two assays observed in our study. Thus, the LA seems to be less sensitive than the LiPA if a sample has a single infection with some specific HPV genotypes that are poorly amplified by PGMY. Even though the majority of samples were cytologically classified as normal, proper HPV assessment, including genotyping, remains essential, particularly for healthy women with normal cytology (35), especially since Wallin and colleagues observed a strong concordance between the HPV type found in baseline smears with normal cytology and the eventual type found in histological samples of invasive cancers (37). In the present study, hr-HPV 51 was missed by LA in four of the discordant cases; this genotype accounted for approximately 0.9% of all squamous cell cervical cancers in previous studies (27). Curiously, the

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inclusivity level for HPV 51 is lower than the level for HPV 16 using PGMY primers, suggesting highly sensitive detection (see the product insert for the CE-marked Linear Array HPV genotyping test, European market). The observed difference in HPV 51 detection between the two assays thus cannot be explained by a lower efficiency of the Linear Array PGMY primer.

After discrepancy analysis of the compatible and discordant cases, both LiPA and LA detected more concordance (Table 6). Some previously undetected genotypes, for example, appeared in the second test run, and vice versa. This could be due to low copy numbers or to sampling, as DNA reextracts were used for the analysis. Also, it could possibly indicate the suggested competition between genotypes present in more or less molar excess. However, results from a discrepancy analysis should generally be handled with care and interpreted carefully. Discrepancy analyses are not perfect, since an analysis is easily biased in favor of the new test, and hard and fast rules do not exist (23). Moreover, the interpretation of results that cannot be dichotomized (i.e., concordant, compatible, and discordant) is less straightforward.

Failing to detect genotypes will lead to underestimation of the prevalence of certain genotypes and will cause false-negative results. Studies concerning (i) the epidemiology of HPV, (ii) HPV vaccination/surgical treatment trials, and (iii) cervical cancer screening and triage, especially, will be negatively affected by this. In epidemiologic studies, genotyping is compulsory in order to evaluate type-specific HPV DNA prevalence among infected women (3), to assess geographic heterogeneity in HPV type distributions (8), and to study type-specific HPV concordance between sexual partners (4). The importance of suitable algorithms for HPV detection and genotyping, in addition to the introduction of type-specific antiviral therapies or monovalent vaccines, was already addressed by Koutsky and colleagues (19). Moreover, current extensive trials testing multivalent vaccines, comprising multiple commonly occurring HPV types, demand accurate, unequivocal, and sensitive methods and algorithms detecting and specifically genotyping HPV (14, 19, 22). These algorithms are also compulsory for clinical trials monitoring surgical treatment of HPV-induced CIN lesions (24, 26) or monitoring persistent infections in consecutive smears, because persistence has been identified as an important risk factor (10, 28). Finally, according to Snijders and colleagues, adding general hr-HPV testing could be beneficial for the efficacy of the population-based screening programs for cervical cancer (32). Castle and colleagues, however, observed that ASCUS women infected with hr-HPV 16 had a 2-year cumulative absolute risk for developing CIN of at least grade 3 of 32.5% compared to the 8.4% risk of developing CIN of at least grade 3 for other high-risk HPV types (6). This underlines the potential importance of assessing the specific genotype causing the HPV infection. Triaging patients using cytology and genotyping assays might have a cost benefit over cytology combined with hr-HPV testing alone. The existence of triage management of ASCUS women in the United States depends solely on an accurate genotyping test (1). Both tests assessed in the present study could be suitable as triage tests.

In addition to accurate genotyping, the appropriate detection of multiple infections seems to be an important application of tests when they are implemented into any format of population-based screening for the prevention of cervical cancer, especially since the presence of multiple human papillomavirus genotypes in a single sample—suggesting repetitive exposure—is suspected to be associated with an increased risk for progressive disease (2). Moreover, mixed infections appear to be more frequent than previously suspected; 35% of the HPV-positive samples and more than 50% of human immunodeficiency virus-positive women are infected with multiple HPV types (12, 21). Multiple infections were less prevalent in cervical carcinomas (15).

In conclusion, the two genotyping assays are handled equally well and have been shown to be highly comparable. All of the HPV genotypes detected in either one or both of the assays, regardless of the analytical or clinical sensitivity and specificity of the tests, should not be trivialized, since their natural behaviors and cancerous potentials in both single and mixed infections remain ambiguous.

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