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Evaluation of Different Techniques for Identification of Human Papillomavirus Types of Low Prevalence▽

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Human papillomaviruses (HPVs) have been recognized as etiologic factors in a variety of diseases. Due to the large number of HPV types, methods for HPV genotyping are difficult to standardize. Despite this fact, several methods exist, and some of them are available commercially. In this study, we evaluated the Roche Diagnostics linear array (LA) HPV genotyping assay, the Innogenetics INNO-LiPA (line probe assay [LiPA]), and two noncommercial reverse line blot (RLB) assays based on either primers GP5+ and GP6+ (GP) or newly designed broad-spectrum primers BSGP5+ and BSGP6+ (BS). The reliabilities of these assays were tested with a wide spectrum of HPV types less prevalent in cervical samples. This is the first study to compare the performance of the most widely used HPV genotyping methods with selected samples positive for low-prevalence HPV types. We focused on interassay agreement, both overall and type specific, in cases with single and/or multiple HPV infections. Interassay agreement was moderate in cases of single HPV infections and poor in cases of multiple HPV infections. The LA and the BS-based RLB assays found a higher rate of cases positive for multiple HPV types than LiPA and the GP-based RLB assay. The weakest capability in detecting multiple HPV infections was observed for LiPA. The use of only one assay in epidemiological and clinical studies might lead to biased conclusions. Therefore, a universally evaluated and agreed upon HPV typing assay or a combination of current assays is needed for possible clinical applications, and knowledge of their limitations is advised.

Human papillomaviruses (HPVs) have been recognized as etiologic factors in cervical carcinoma, precancerous lesions of the cervix uteri, and several other anogenital cancers in females and males (for reviews, see references 3 and 24). In addition, about 26% of head and neck cancers are linked to HPV infection (13). HPVs represent an extremely heterogeneous group of DNA viruses. Until now, more than 100 HPV types have been identified and fully sequenced (9). Approximately 40 HPV types infecting the anogenital epithelium are classified as either low risk (LR) or high risk (HR) on the basis of their oncogenic potentials. A recent meta-analysis has designated 15 anogenital HPV types as HR, with an additional 3 HPV types designated as probable HR types (23).

Because of their biological properties, HPVs cannot easily be grown in tissue culture, which makes the preparation of antigens for the routine detection of HPV difficult (10). Furthermore, serological assays, which detect HPV-specific antibodies, cannot be used for diagnostic purposes, since these antibodies are markers of a lifetime's cumulative exposure to HPV types (18). For diagnostic purposes, methods based on the detection of HPV-specific nucleic acids are being used. In addition, the typing of HPV isolates is done by means of molecular biological methods (4).

Due to the large number of HPV types, methods for HPV genotyping are difficult to standardize. Despite this fact, several methods exist, and some of them are available commercially. These HPV typing methods are mostly based on PCR techniques with degenerate and/or consensus primers, followed by an additional assay for the type-specific identification of HPV. The most widely used PCR methods for the detection of mucosal HPV types are based on primer set MY09-MY11 (21), GP5+-GP6+ (8), or SPF10 (19). All these primer sets target the L1 region of the HPV genome. Several authors have reported on the efficiency of amplification with these primers as well as on a comparison of method performance (29, 34). The GP5+-GP6+-based PCR system seems to be more sensitive, more reliable, and reproducible than the MY09-MY11-based PCR system (26). Additionally, primers PGMY09 and PGMY11 (14), adapted from primers MY09 and MY11, detect genital HPV types at a higher rate than primers MY09 and MY11 (7). In general, HPV typing assays have good agreement when a infection with a single HPV type is present, but agreement is lower when infections with multiple HPV types are present (12).

In this study, we evaluated the Roche Diagnostics linear array (LA) HPV genotyping assay (5), the Innogenetics INNO-LiPA (line probe assay [LiPA]) (20), and the noncommercial GP5+-GP6+-based reverse line blot (GP-RLB) assay (35). Additionally, an improved RLB assay was performed with the broad-spectrum primers BSGP5+ and BSGP6+ (BS) designed by Schmitt et al. (29), the BS-RLB assay. All of these assays are well suited for population-based epidemiological studies due to their easy and fast protocols. In this study, their reliabilities were tested with HPV types less frequently present

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in individuals infected with multiple HPV types. To our knowledge, this is the first study to compare the performance characteristics of the most widely used HPV genotyping methods with selected samples positive for low-prevalence HPV types.

MATERIALS AND METHODS

Sample collection. A total of 153 samples from two countries, Croatia and the Czech Republic, were used for this study. The first set of samples consisted of 86 cervical DNA samples from Croatian women with histologically confirmed high-grade squamous intraepithelial lesions (HSILs) (30). The samples were selected from a collection of DNA samples previously collected and screened for HPV DNA by the MY09-MY11-based PCR followed by type-specific PCR detection of HPV types 6/11, 16, 18, 31, and 33 (15, 16). The selected samples were positive by the MY09-MY11-based PCR but were not positive for HPV type 6, 11, 16, 18, 31, or 33 by type-specific primer-directed PCRs. In this study, the HPV types in the selected samples were analyzed in parallel by the LA assay, LiPA, and the GP- and BS-RLB assays.

The second set of samples consisted of 40 DNA samples from women from the Czech Republic with HSILs and 27 samples from women with low-grade squamous intraepithelial lesions (LSILs) (31). The samples were chosen on the basis of typing by GP5+-GP6+-based PCR followed by the GP-RLB assay. Twenty samples infected with single HPV types other than HPV types 6, 11, 16, 18, 31, and 33 were selected. Additionally, 47 samples infected with multiple HPV types, i.e., with more than one HPV type, were chosen irrespective of the HPV type 6, 11, 16, 18, 31, and 33 infection status. All samples were further tested by the LA assay, LiPA, and the BS-RLB assay.

LiPA. The LiPA from Innogenetics uses biotinylated SPF10 PCR primers for the amplification of a 65-bp region of the L1 gene of a broad spectrum of HPV types (22). LiPA is capable of detecting 26 HPV types (Table 1). The assay was performed according to the manufacturer's protocol. Briefly, PCR was performed in a total volume of 50 μl containing 5 μl of 10× PCR buffer, 200 μM of each deoxynucleoside triphosphate, 10 μl primer mix, 2 mM $MgCl_2$, 1.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), and 10 μl (approximately 250 ng) of DNA. The DNA amplification was performed as follows: 9 min of denaturation at 94°C; 40 cycles of 30 s of denaturation at 94°C, 45 s of annealing at 52°C, and 45 s of elongation at 72°C; and a final extension for 5 min at 72°C. After amplification, 10 μl of the PCR product was denatured with the denaturation solution provided by the manufacturer and hybridized with oligonucleotide probes immobilized on strips. After a stringent wash, hybrids were visualized by the addition of streptavidin-alkaline phosphatase conjugate, which binds to the biotinylated PCR primers, and a substrate (5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium) that generates a purple precipitate at the probe line.

LA assay. The LA assay from Roche Diagnostics uses the same principles as LiPA, but with different PCR primers and oligonucleotide probes (5). The LA assay uses the PGMY09-PGMY11 primer set, which amplifies a 450-bp fragment of the L1 gene, and is capable of detecting 37 HPV types (Table 1). The assay was performed according to the manufacturer's instructions. Briefly, DNA was amplified in a total volume of 100 μl containing 50 μl (approximately 500 ng) of sample DNA and 50 μl of the master mixture provided by the manufacturer. The amplification protocol was as follows: 9 min of denaturation at 95°C and 40 cycles of 30 s of denaturation at 95°C, 1 min of annealing at 55°C, and 1 min of elongation at 72°C, followed by a final extension for 5 min at 72°C. After amplification, the whole PCR product was denatured and hybridized with oligonucleotide probes immobilized on strips. After a stringent wash, the hybrids were detected by the addition of streptavidin-horseradish peroxidase conjugate, which binds to the biotinylated PCR primers, and a substrate (hydrogen peroxide and 3,3′,5,5′-tetramethylbenzidine) that generates a purple precipitate at the probe line.

GP-RLB assay. The RLB hybridization assay enables the detection of HPV and genotyping of 37 different HPV types (Table 1) (32). HPV detection was performed by PCR with primer GP5+ and with primer GP6+ labeled with biotin at the 5′ end; these two primers generate a 150-bp fragment of the L1 gene. The PCR was performed for 40 cycles, with each cycle consisting of 90 s of denaturation at 94°C, 30 s of annealing at 38°C, and 80 s of elongation at 71°C. Defined ramping times were used. The first cycle was preceded by a 5-min denaturation step at 94°C, and the last cycle was followed by incubation at 71°C for 4 min. Oligonucleotide probes with a 5′-terminal amino group are covalently linked to an activated negatively charged Biodyne C membrane with a miniblotter (MN45; Immunetics, Boston, MA). For hybridization with PCR products, the membrane is rotated 90° and samples are applied with a clean miniblotter so that the sample lines are perpendicular to the probe lines. After a stringent wash, the membrane is incubated with streptavidin-horseradish peroxidase conjugate. The conjugate was visualized with an enhanced chemiluminescence detection liquid system (Amersham) and by exposure of the membrane to LumiFilm (Roche) for 5 min.

BS-RLB assay. The BS-RLB analyses were performed as described above for the GP-RLB assay, and the BS-based PCR was conducted as reported previously (29). Briefly, eight additional forward primers and two additional 5′-biotinylated backward primers were added to the GP-PCR mixture: 200 nM of each forward primer (including primer GP5+), 400 nM of each backward primer (including

TABLE 1. Genotyping capabilities of the different assays used in this study

Oncogenic potential[a] and HPV type	Genotyping capability			
	LA assay (Roche)	LiPA (Innogenetics)	GP-RLB assay	BS-RLB assay
LR				
6	√	√	√	√
11	√	√	√	√
34		√	√	√
40	√	√	√	√
42	√	√	√	√
43		√	√	√
44		√	√	√
54	√	√	√	√
55	√		√	√
57			√	√
61	√		√	√
62	√			
64	√			
67	√			
69	√			
70	√	√	√	√
71	√		√	√
72	√		√	√
74		√		
81	√		√	√
83	√		√	√
84	√		√	√
IS39	√		√	√
89	√		√	√
HR				
16	√	√	√	√
18	√	√	√	√
31	√	√	√	√
33	√	√	√	√
35	√	√	√	√
39	√	√	√	√
45	√	√	√	√
51	√	√	√	√
52	√[b]	√	√	√
56	√	√	√	√
58	√	√	√	√
59	√	√	√	√
68	√	√[c]	√	√
73	√	√[c]	√	√
82	√		√	√
Probably HR				
26	√		√	√
53	√	√	√	√
66	√	√	√	√
No. of detectable HPV types	37	26	37	37

[a] According to Muñoz et al. (23).
[b] The LA assay cannot distinguish HPV-52 in samples containing HPV type 33, 35, or 58.
[c] LiPA cannot distinguish HPV types 68 and 73 from each other.

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the LA assay cannot exclude HPV type 52 when it is present together with HPV type 33, 35, or 58 and LiPA cannot distinguish HPV types 68 and 73 from each other, as described in the respective manufacturers' protocols. For all samples for which one of those types could not be clearly excluded due to the presence of confounding types, the sample was considered positive by the assay. This is a known limitation of both assays and was not corrected for in any calculation in any way to make the evaluation as objective as possible.

The prevalence of specific HPV types as detected by the LA assay, LiPA, the GP-RLB assay, and the BS-RLB assay is shown in Table 2. The frequencies of each HPV type when it was found in each sample by at least one assay or all four assays are also indicated in Table 2. The most common HPV type in the samples analyzed was HPV type 58 (HPV-58), which was identified by all four assays in 21 of the 153 (13.7%) cases. The next most abundant HPV types were HPV-16, found by all assays in 17 (11.1%) samples, and HPV-51, found by all assays in 16 (10.5%) samples. HPV types 52 and 66 were both found in 15 of 153 (9.8%) cases. HPV types 45, 56, and 31 were found in 14 of 153 (9.2%), 13 of 153 (8.5%), and 9 of 153 (5.9%) samples, respectively. The frequency of other HPV types was below 5%. HPV types 57, 64, 69, and 74 were not found in any of the samples tested by any assay. The overall typing capability of the LA assay was the best (99.3%), followed by the BS-RLB assay (97.4%), LiPA (95.4%), and the GP-RLB assay (94.8%) (Table 2).

By means of the LA assay, LiPA, the GP-RLB assay, and the BS-RLB assay, HPV types detectable by all four assays (assay common types) were found in 94.1%, 92.8%, 88.2%, and 94.1% of the 153 samples, respectively; and multiple infections were detected by the four assays in 55.6%, 37.3%, 43.1%, and 52.9% samples, respectively (Table 3). The majority of infections with multiple HPV types found were double infections: 58.8%, 54.4%, 66.7%, and 60.5% by the LA assay, LiPA, the GP-RLB assay, and the BS-RLB assay, respectively. The highest number of HPV types in a single sample was identified by the LA assay, with a mean of 2.5 HPV types per sample (range, 1 to 8 HPV types per sample); the BS-RLB assay detected a mean of 2 HPV types per sample (range, 1 to 6 HPV types per sample); and 1.7 HPV types per sample were identified by both LiPA and the GP-RLB assay (ranges, 1 to 6 HPV types per sample for LiPA and 1 to 4 HPV types per sample for the GP-RLB assay). Fisher's exact test showed that for assay common HPV types, the LA and BS-RLB assays were the most sensitive in detecting infections with multiple HPV types (LiPA versus LA assay, $P = 0.0015$; LiPA versus BS-RLB assay, $P = 0.0067$). The GP-RLB assay detected fewer multiple infections than the LA assay ($P = 0.0940$) and the BS-RLB assay ($P = 0.2320$), but this was not significant in either case. There was no significant difference between the LA and the BS-RLB assays ($P = 0.7206$) or between LiPA and the GP-RLB assay ($P = 0.1488$).

Figure 1 compares the frequencies of the HR and the LR HPV types detected by the four assays. The HPV types are listed in decreasing frequency of detection by any assay. Figure 1 indicates the assay common HPV types for which Friedman's test showed a statistically significant discrepancy between the assays tested. These were for HPV types 16 ($P = 0.009$), 39 ($P = 0.0097$), 42 ($P < 0.0001$), 51 ($P = 0.0044$), 52 ($P < 0.00001$), 53 ($P < 0.00001$), 54 ($P = 0.006$), 56 ($P = 0.0004$), 58 ($P = 0.0007$), 59 ($P = 0.0293$, 68 ($P = 0.0009$), and 73 ($P = 0.062$).

TABLE 3. Types of HPV infections present in samples, identified by LA assay (Roche), LiPA (Innogenetics), GP-RLB assay, and BS-RLB assay, and common to all four assays

No. of HPV types[a]	No. (%) of cases detected by:			
	LA assay[b]	LiPA[c]	GP-RLB assay	BS-RLB assay
Any	144 (94.1)	142 (92.8)	135 (88.2)	144 (94.1)
Single	59 (38.6)	85 (55.6)	69 (45.1)	63 (41.2)
Multiple	85 (55.6)	57 (37.3)	66 (43.1)	81 (52.9)
Two	50 (32.7)	31 (20.3)	44 (28.8)	49 (32.0)
Three	16 (10.5)	17 (11.1)	18 (11.8)	18 (11.8)
Four	11 (7.2)	7 (4.6)	4 (2.6)	10 (6.5)
Five	7 (4.6)	1 (0.7)	0 (0)	4 (2.6)
Six	1 (0.7)	1 (0.7)	0 (0)	0 (0)
None	9 (5.9)	11 (7.2)	18 (11.8)	9 (5.9)

[a] HPV types present in all four assays: HPV types 6, 11, 16, 18, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, and 73.
[b] The LA assay cannot distinguish HPV-52 in samples containing HPV type 33, 35, or 58.
[c] LiPA cannot distinguish HPV types 68 and 73.

The interrate agreement (κ value) and the 95% confidence interval were calculated for all assay pairs for all HPV types (data not shown). In some cases, it was not possible to calculate the κ value, as some HPV types were not present in either of the pair of assays or no cases of that HPV type were found in a particular pair. The agreement between assays was calculated as the average agreement in detecting all HPV types common for that assay pair. The best assay pair agreement was observed between the LA assay and LiPA (average κ value = 0.68), followed by the GP-RLB and the BS-RLB assays (average κ value = 0.67), the LA and the BS-RLB assays (average κ value = 0.62), and LiPA and the BS-RLB assay (average κ value = 0.66), all of which indicate good strengths of agreement. Moderate agreement strengths with average κ values of 0.54 and 0.57 were obtained for the LA and the GP-RLB assays and for LiPA and the GP-RLB assay, respectively (data not shown).

The strength of interassay agreement for each HPV type was calculated as an average of all interassay pair κ values for a particular type. It was calculated only for assay common types. Figure 2 presents these results for each HPV type in decreasing order of strength of agreement. HPV-35 had the greatest average κ value (0.94) and, along with HPV types 11, 45, 51, 56, 58, 66, and 70, fits into the very good agreement group (range of κ values for these types, 0.82 to 0.94). The interassay agreement for HPV types 16, 18, 31, 33, 39, 42, and 68 was good (range of κ values, 0.61 to 0.79). Moderate interassay agreement (range of κ values, 0.54 to 0.58) was obtained for HPV types 6, 52, 53, 59, and 73. The assays had a fair strength of interassay agreement for HPV type 54 (κ value, 0.27). The interassay agreement for HPV-40 was worse than chance, having an average κ value of −0.002. For the other HPV types, the interassay agreement could not be determined.

Table 4 summarizes the agreement between assays for the typing of assay common types in cases of single versus multiple infections. Thus, in 71.8% cases of single infections, all four assays agreed on the types present, while in cases of multiple

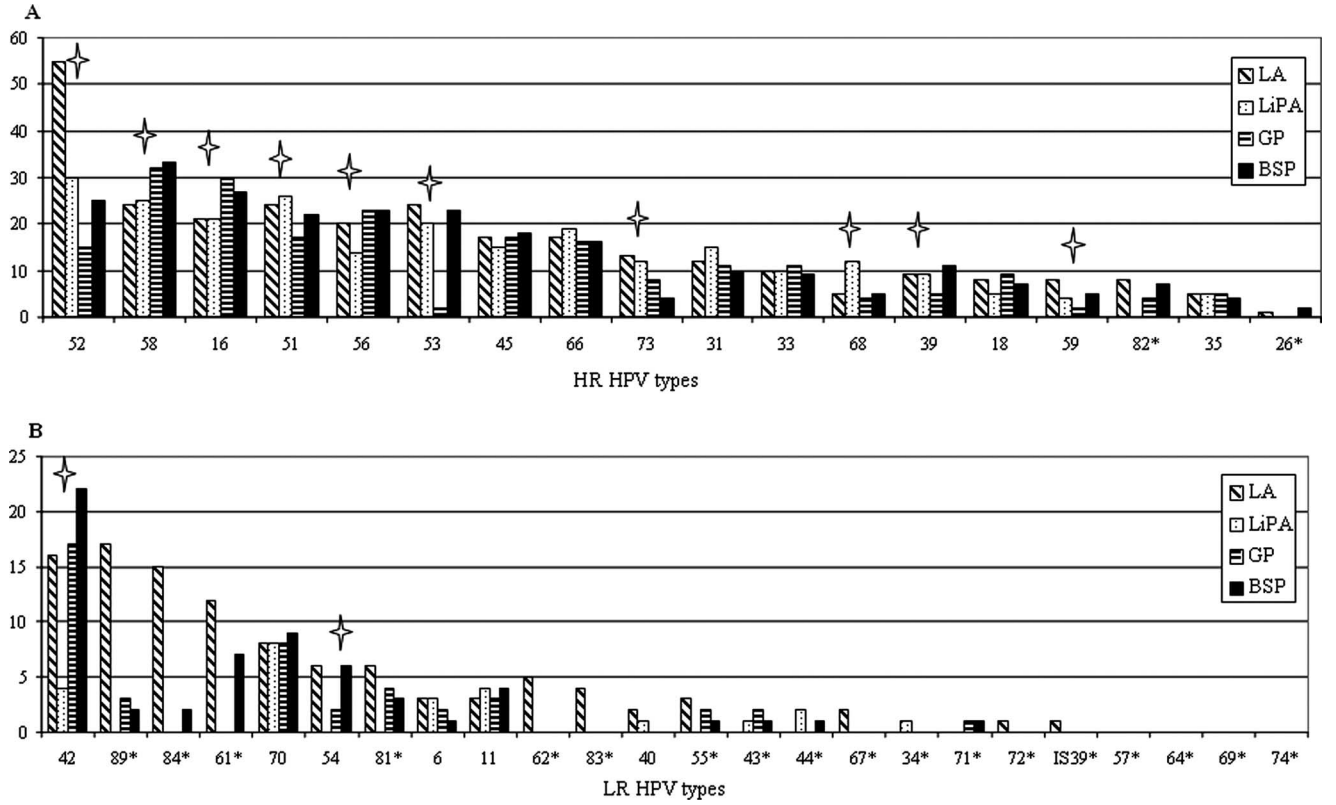

FIG. 1. Frequencies of detection of HR (A) and LR (B) HPV types by the LA assay, LiPA, the GP-RLB assay, and/or the BS-RLB assay in 153 selected samples; HPV types are sorted by decreasing average frequency as detected by any of the assays; stars, significant differences (Friedman test) between assays for a particular HPV type; *, HPV types not included in all four assays (Table 1); the LA assay cannot distinguish HPV-52 in samples containing HPV type 33, 35, or 58; and LiPA cannot distinguish HPV types 68 and 73 from each other.

infections, complete agreement was found in only 6.2% cases, and in 93.8% of samples with multiple infections, partial agreement was found between the four assays. Agreement was considered complete if all four assays gave completely identical results for the sample in question on the basis of assay common types. On the other hand, agreement was considered partial when any two or more assays agreed on one or more of the HPV types present in a particular sample. The values were comparable when we evaluated all types identifiable by any assay and not only assay common types (data not shown).

DISCUSSION

The reason for this study was the lack of published data on comparisons of test performances for low-prevalence HPV types.

All four assays, the LA assay, LiPA, the GP-RLB assay, and the BS-RLB assay, evaluated in this study enabled the detection of up to 16 HR or possible HR HPV types, depending on the assay, which justifies their use for epidemiological and clinical studies. Additionally, the LA assay and both RLB assays also allowed the detection and typing of more LR HPV types than LiPA did (Table 1). This advantage of the LA, GP-RLB, and BS-RLB assays can be interesting for epidemiological studies; i.e., some low-abundance LR types might be shown to be associated with neoplastic changes in cervical squamous cells. For instance, in our study LR HPV-70 as a single infection was found unexpectedly often in HSIL samples (data not shown); larger epidemiological studies are necessary to possibly classify it among the HR HPV types.

Considering all types that an assay is able to detect, the LA assay was the most sensitive, as it was able to reveal the HPV type present in all except one sample. For the BS-RLB assay, LiPA, and the GP-RLB assay, the numbers of samples in which HPV was detected were slightly lower for assay common HPV types only.

The highest number of HPV types in a single sample was identified by the LA assay, followed by the BS-RLB assay, LiPA, and the GP-RLB assay. This could indicate the low sensitivity of LiPA and the GP-RLB assay or an unspecific amplification and/or hybridization by the LA and BS-RLB assays. For assay common HPV types, the LA and BS-RLB assays were the two assays that detected multiple HPV infections (more than one HPV type in a sample) with the most sensitivity, while the least sensitive assay was LiPA. However, we can hypothesize that these data show that LiPA is the most specific assay, while the LA and BS-RLB assays reveal a certain level of unspecific amplification and/or hybridization. This large difference in multiple HPV infections identified could not be attributed to the fact that the RLB and LA assays tested for more HPV types than LiPA did, as only assay common types were considered. This observation was in disagreement with the results of Gillio-Tos et al. (12), who have reported that

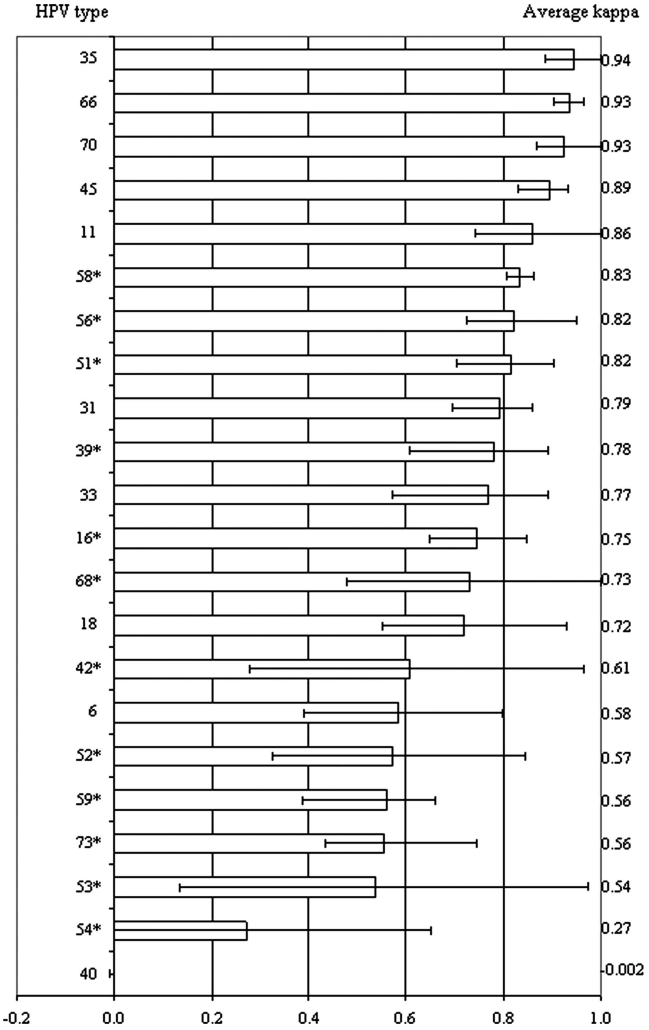

FIG. 2. Average κ value of one-by-one interassay agreement measurements for each HPV type present by the LA assay, LiPA, the GP-RLB assay, and the BSP-RLB assay. *, HPV types for which significant differences were observed; the LA assay cannot distinguish HPV-52 in samples containing HPV type 33, 35, or 58; LiPA cannot distinguish HPV types 68 and 73.

LiPA is more sensitive than the GP-RLB and Amplisense assays.

The strength of the interassay agreement for each assay common type was calculated as the average κ value of all assay pair combinations for that type (data not shown). It is interesting to note that only good agreement and not a perfect strength of agreement was found for the most common HR HPV types (types 16, 18, 31, and 33). This observation suggests a possible problem for accurate typing. Furthermore, significant differences were observed in 12 of 22 HPV types common to all four assays. For HPV-58, differences arose from the lower number of positive samples detected by the LA assay and LiPA compared with the number of positive samples detected by both versions of the RLB assay, although the total agreement for HPV-58 still indicated very good agreement. The same observation was found for HPV types 56 and 51; HPV-51 was detected the least often by the GP-RLB assay and the most often by LiPA, while HPV-56 was found the least often by LiPA (Table 2; Fig. 1).

HPV-45 was among the HPV types for which the assays had very good agreement, and no significant discrepancy was observed; it was detected only slightly less often by LiPA.

Significant interassay variations were revealed for HPV types 39, 42, and 68, as their average κ values indicated only good agreement. For HPV-39, the GP-RLB assay gave the fewest number of positive samples. In the case of HPV-42, the BS-RLB assay gave the most positive results and LiPA missed most of the positive samples. The low sensitivity of LiPA for HPV-42 was described previously (34). For HPV-68, LiPA gave a great overestimation, as it failed to discriminate HPV 68 and 73, but even with this known limitation (27), the agreement between assays was quite good. Nevertheless, this overestimation will pose problems if LiPA is used alone in epidemiological studies and especially if these two types have different prevalences in the population studied.

Differences between assays for the typing of the most common LR HPV types, types 6 and 11, were not significant for either type; but the strengths in agreement between the assays were very different for these types. Since our sample pool was artificially depleted of these types, the result is of limited value. In contrast, the highest strength of interassay agreement was found for HPV types 35, 66, and 70, indicating that these types are almost equally detected by all four assays.

Moderate agreement was also observed for HPV types 52, 53, 59, and 73. This might pose a bigger problem, as all these types are HR HPV types, and for each of them the discrepancy was significant. For HPV-52, the LA assay grossly overestimated its presence, as it cannot distinguish it from HPV type 33, 35, or 58 (6). As a consequence, HPV-52 was the most common type found by this assay. All other assays detected HPV-52 less often, with the GP-RLB assay having the lowest rate of detection of this type. We stress again that this overestimation is due to the LA assay design and is a known

TABLE 4. Overview of interassay agreement between LA assay, LiPA, GP-RLB assay, and BSP-RLB assay regarding their capability for HPV type identification

Type of infection	HPV types present in all four assays[a]		Agreement[b]
	No. (%) of samples	No. of cases (% of group)	
All	152 (99.3)	35 (23.0)	Complete
		111 (73.0)	Partial
		6 (3.9)	None
Single	39 (25.5)	28 (71.8)	Complete
		5 (12.8)	Partial
		6 (15.4)	None
Multiple	113 (73.9)	7 (6.2)	Complete
		106 (93.8)	Partial
		0	None

[a] The LA assay cannot distinguish HPV-52 in samples containing HPV type 33, 35, or 58; and LiPA cannot distinguish HPV types 68 and 73. One sample (0.7%) had no HPV type present by all four assays.

[b] Complete, identical HPV types were simultaneously detected by all four assays in one sample; partial, one or more HPV types were in common by two or more assays; none, different HPV types were detected by all four assays.

limitation of this assay. Until this limitation is overcome, samples indicated to contain HPV-52 by the LA assay should be viewed with greater caution in epidemiological studies. On the other hand, when clinical samples are evaluated for diagnostic purposes, this probe cross-reactivity is not as important, as the patient has already been found to be infected with an HR HPV type (type 33, 35, or 58) and, regardless of HPV-52 positivity, should be considered at higher risk of disease. The difference observed for HPV-53 had to be attributed primarily to the fact that the GP-RLB assay failed to detect this type, as has been shown earlier by Qu et al. (25). In the case of HPV-73, we expected the same problem with LiPA that was encountered with HPV-68. However, when the LiPA findings for HPV-73 are compared with the LA assay findings, LiPA slightly underestimated this type's presence, while the BS-RLB assay greatly underestimated its presence in comparison with the prevalence of this type detected by LiPA or the LA assay.

The only HPV type which showed a fair strength of interassay agreement was HPV-54. In this case, LiPA completely failed to detect it, while the GP-RLB assay found it less often than the remaining two assays.

HPV-40, which can be typed by all four assays, was detected in only two cases by the LA assay and once by LiPA, but not in the concordant sample (Table 2). Even though the number of HPV-40-positive samples was very low, this result suggests that the primer and probes used in both RLB assays should be reevaluated and/or the assays should be compared by use of a larger pool of HPV-40-positive samples.

Complete agreement between assays in detecting assay common HPV types in single and multiple infections was the highest in cases of single infections, but so was complete disagreement. In cases of multiple infections, partial agreement dominated; and because of this, when all types of infections were combined, partial agreement was again the most common. This clearly indicates that the LA assay, LiPA, the GP-RLB assay, and the BS-RLB assay do not in reality give the same answer when they are used to test samples infected with multiple HPV types; and when such infections are common in the study population, this greatly affects overall assay agreement. On the other hand, the assays are not likely to completely disagree (Table 4). Similar results were observed by Gillio-Tos et al. (12), who compared the GP-RLB assay, LiPA, and the Amplisense assay and reported high levels of assay concordance for single infections but limited concordance for multiple infections. This is probably due to the differences in the affinities of the primer sets for the different HPV types. In addition, the concentration of a particular HPV type in the sample plays a role. In our experience, the use of a combination of typing methods only with knowledge of the limitation of each of them allows the unambiguous HPV typing, but the cost is unacceptable for routine settings.

When the results of similar studies are compared to our results, both van Hamont et al. (36) and van Doorn et al. (35), who compared only the LA assay and LiPA, have found these two assays to be highly comparable for the detection of assay common HPV types. While in our study these assays showed a higher average κ value than the other assay pairs, we found their strengths of agreement to be good, although not very good. Contrary to our study, both of those studies were designed not to be challenging for the assays and usually analyzed samples with normal cytologies, a relatively small number of samples infected with multiple HPV types, and a normal distribution of HPV types. In our study, we wanted to evaluate the assays under more difficult conditions when low-prevalence and/or multiple HPV types were present. In such a sample pool, there is a strong possibility that different HPV types compete for the PCR reagents, and thus, especially when the HPV types are not present in the same amounts, the levels of the PCR amplicons of one type might remain below the detection limit of the assay.

Some authors (1, 2, 22, 28, 33) have found infections with multiple HPV types to be associated with a higher risk of progressive disease or cervical neoplasia, while some (22) have described fewer multiple infections in women with cervical cancer or a decreasing number of HPV types with an increasing severity of disease (34). In addition, some authors have reported no increased risk of cervical intraepithelial neoplasia or cervical cancer among women with multiple HPV infections than among women with single HPV infections (3, 17), while others have found a discrepant result (11). On the basis of our results, it is clear that to strongly link infection with multiple HPV types with disease progression, the choice of typing assay is essential.

In this study, we have considered the ability of each of the four assays tested to detect a wide spectrum of less prevalent HPV types. We focused on interassay agreement, both overall and type specific, in cases with single and/or multiple HPV infections. Our results showed a large variability in the ability of a particular assay to detect different HPV types. The LA and BS-RLB assays found larger numbers of cases positive for multiple types than the two other assays did. The lowest capability of detecting multiple infections was observed for LiPA. The interassay agreement was moderate for single infections and poor for multiple infections. The use of only one assay in epidemiological and clinical studies might lead to biased conclusions. Therefore, a universally evaluated and agreed upon HPV typing assay or a combination of current assays is needed for possible clinical applications, and knowledge of their limitations is advised.

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