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# EVALUATION OF DIFFERENT TECHNIQUES FOR THE IDENTIFICATION OF HUMAN PAPILLOMAVIRUS (HPV) TYPES OF LOW PREVALENCE

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## Abstract

Human papillomaviruses (HPV) have been recognized as an etiologic factor 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 (LiPA) assay and two non-commercial Reverse Line Blot (RLB) assays based on either GP5+/6+ primers (GP) or newly designed Broad-Spectrum Primers (BS). The reliability of the above-mentioned assays was tested on a wide spectrum of less prevalent HPV types in cervical samples. This is the first study to compare the performance of the most widely used HPV genotyping methods on selected samples positive for low prevalent HPV types. We focused on inter-assay agreement, both overall and type-specific, in cases with single and/or multiple HPV infection. Inter-assay agreement was moderate in single HPV infections and poor in multiple HPV infections. LA and BS-RLB found a higher rate of cases positive for multiple HPV types than LiPA and GP-RLB. The lowest capability in detecting multiple HPV infections was observed for the LiPA assay. 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 is needed for possible clinical applications, or a combination of current assays, with a knowledge of their limitations, is advised.

## Introduction

Human papillomaviruses (HPV) have been recognized as an etiologic factor in cervical carcinoma, precancerous lesions of the cervix uteri and several other anogenital cancers in females and males (for a review see 3, 24). In addition, about 26% of head and neck cancers are linked to HPV infection (13). HPV 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) based on their oncogenic potential. A recent meta-analysis has designated 15 anogenital HPV types as HR, with an additional 3 HPV types designated as probable HR (23).

Because of their biological properties, HPV 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 using degenerate and/or consensus primers, followed by an additional assay for type-specific identification of HPV. The most widely used PCR methods for the detection of mucosal HPV types are based on MY09/11 (21), GP5+/6+ (8) or SPF10 (19) primer sets. All these primers target the L1 region of the HPV genome. Several authors report on the efficiency

of amplification with these primer sets as well as on a comparison of method performance (29, 34). The GP5+/6+ PCR system seems to be more sensitive, more reliable and reproducible when compared to MY09/11 (26). Additionally, the PGMY09/11 primers (14), adapted from MY09/11 primers, have a higher detection rate compared to MY09/11 for genital HPV types (7). In general, HPV typing assays have good agreement when a single infection is present, but agreement is lower for infections with multiple HPV types (12).

In this study, we evaluated the Roche Diagnostics Linear Array (LA) HPV genotyping assay (5), the Innogenetics INNO-LiPA (LiPA) assay (20) and the non-commercial GP5+/6+-based Reverse Line Blot assay (GP-RLB) (35). Additionally, an improved RLB assay was performed using Broad-Spectrum Primers (BS) designed by Schmitt et al. (29). All of the above-mentioned assays are well suited for population-based epidemiological studies due to their easy and fast protocol. In this study, their reliability is tested on less frequently present HPV types in an infection containing multiple HPV types. This is the first study to our knowledge that compares the performance of the most widely used HPV genotyping methods on selected samples positive for low prevalent 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 (HSIL) (30). The samples were selected from a collection of DNA samples

86 previously collected and screened for HPV DNA using MY09/11-based PCR followed by
87 type-specific PCR detection of HPV types 6/11, 16, 18, 31 and 33 (15, 16). The selected
88 samples were positive for MY09/11 PCR but were not positive for HPV types 6, 11, 16,
89 18, 31 or 33 by type-specific primer directed PCR. In this study, the HPV types of the
90 selected samples were analyzed in parallel by LA, LiPA, GP- and BS-RLB assays.

91 The second set of samples consisted of 40 DNA samples from women with HSIL
92 and 27 samples from women with Low-grade Squamous Intraepithelial Lesions (LSIL)
93 from the Czech Republic (31). The samples were chosen on the basis of typing by
94 GP5+/6+-based PCR followed by a GP-RLB assay. Twenty samples containing single
95 HPV infection with types other than HPV 6, 11, 16, 18, 31 and 33 were selected.
96 Additionally, 47 samples containing multiple infections, i.e. with more than one HPV
97 type, were chosen irrespective of HPV 6, 11, 16, 18, 31 and 33 statuses. All samples were
98 further tested by LA, LiPA, BS-RLB assays.

99 *Innogenetics INNO-LiPA (LiPA) assay*

100 The LiPA assay from Innogenetics uses biotinylated SPF10 PCR primers for the
101 amplification of a 65 base pair region of the L1 gene of a broad spectrum of HPV types
102 (22). The LiPA assay is capable of detecting 26 HPV types (Table 1). The assay was
103 performed according to the manufacturer's protocol. Briefly, PCR was performed in a
104 total volume of 50 µl, containing 5 µl of 10X PCR buffer, 200 µM of each dNTP and 10
105 primer mix, 2 mM $MgCl_2$, 1.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems,
106 Foster City, CA, USA), and 10 µl (app. 250 ng) of DNA. The DNA amplification was
107 performed as follows: 9 minutes of denaturation at 94$^{o}$C, 40 cycles of 30 seconds of
108 denaturation at 94$^{o}$C, 45 seconds of annealing at 52$^{o}$C, 45 seconds of elongation at 72$^{o}$C,
109 and a final extension for 5 minutes at 72$^{o}$C. After amplification, 10 µl of PCR product

was denatured with the provided denaturation solution and hybridized with oligonucleotide probes immobilized on strips. After stringent washing, 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), generating a purple precipitate at the probe line.

*Roche Diagnostics Linear Array (LA) assay*

The LA assay from Roche Diagnostics uses the same principles as LiPA, with different PCR primers and oligonucleotide probes (5). LA uses the PGMY09/11 primer set amplifying 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 of 100 µl containing 50 µl (app. 500 ng) of sample DNA and 50 µl of master mix provided by the manufacturer. The amplification protocol was as follows: 9 minutes of denaturation at 95°C; 40 cycles of 30 seconds of denaturation at 95°C, 1 minute of annealing at 55°C, 1 minute of elongation at 72°C followed by 5 minutes at 72°C final extension. After amplification, the whole PCR product was denatured and hybridized with oligonucleotide probes immobilized on strips. After stringent washing, 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), generating a purple precipitate at the probe line.

*Reverse Line Blot assay with GP5/6+ primers (GP-RLB)*

The RLB hybridization assay enables the detection of the presence of HPV and genotyping of 37 different HPV types (Table 1) (32). HPV detection was performed by PCR with primers GP5+ and 5'-end biotin-labeled GP6+, which generate a 150 bp long

fragment of the L1 gene. The PCR was performed for 40 cycles: each cycle consisting of 90 seconds denaturation at 94°C, 30 seconds annealing at 38°C and 80 seconds elongation at 71°C. Defined ramping times were used. The first cycle was preceded by a 5-minute denaturation step at 94°C, and the last cycle was followed by incubation at 71°C for 4 minutes. Oligonucleotide probes with a 5'-terminal amino group are covalently link to an activated negatively charged Biodyne C membrane using a miniblotter MN45 (Immunetics, Boston, MA). For hybridization with PCR products, the membrane is rotated 90° and samples are applied using a clean miniblotter, so sample lines are perpendicular to the probe lines. After stringent washing, the membrane is incubated with streptavidin-horseradish peroxidase conjugate. The conjugate was visualized by the ECL detection liquid system (Amersham) and by exposure of the membrane for 5 minutes to LumiFilm (Roche).

*Reverse Line Blot assay with BS primers (BS-RLB)*

RLB analyses were performed as described above for GP-RLB, and BS PCR was conducted as reported previously (29). Briefly, 8 additional forward and 2 additional 5´-biotinylated backward primers were added to the GP PCR reaction. 200 nM of each forward (including GP5+), 400 nM of each backward (including GP6+) and 300 nM of each of the β-globin primers $MS_3$ and 5´-biotinylated $MS_{10}$ were used (29). Otherwise, the PCR buffers, reagents and temperature profiles were identical to those described above.

*Statistical analysis*

The qualitative hybridization results obtained by different methods were read by eye and entered and processed manually into Microsoft Excel on a personal computer. The performance of different techniques was analyzed using a nonparametric Friedman

158 test and Fischer's exact test (GraphPad Prism version 4.00 for Windows, GraphPad
159 Software, San Diego California USA, www.graphpad.com). Pairs of assays were
160 compared using the unweighted Kappa statistic (MedCalc version 7.3.0.1, MedCalc
161 Software, Mariakerke Belgium, www.medcalc.be). A Kappa (K) value of 0 indicates no
162 agreement better than chance and a value of 1 indicates perfect agreement. K values from
163 0 to 0.20, 0.21 to 0.40, 0.41 to 0.60, 0.61 to 0.80, and 0.81 to 1.00 indicate poor, fair,
164 moderate, good and very good agreement strength, respectively.

165

166 **Results**

167 In this study, 153 cervical DNA samples were analyzed. The whole study
168 consisted of 126 (83%) HSIL and 27 (17%) LSIL samples. All samples were previously
169 typed and were positive for less frequent HPV types. Samples positive for HPV 6, 11, 16,
170 18, 31 and 33 single infection were not included in the selection, but samples with these
171 types in multiple infections with less common types were included.

172 Note that two of the four assays compared have some *a priori* limitations in
173 distinguishing some HPV types, namely, LA cannot exclude HPV type 52 when present
174 together with HPV type 33, 35 or 58, and LiPA cannot distinguish HPV types 68 and 73
175 from each other, as described in the respective manufacturers' protocols. In all samples
176 where one of those types could not be clearly excluded due to the presence of
177 confounding types it was considered to be positive by this assay. This is a known
178 limitation of both assays and was not corrected in any calculation in any way to make the
179 evaluation as objective as possible.

180 The prevalence of specific HPV types as detected by LA, LiPA, GP-RLB and BS-
181 RLB is shown in Table 2. The frequencies for each HPV type when found with at least

one assay per sample or all four assays are also indicated in Table 2. The most common HPV type in the analyzed samples was 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%) and HPV 51 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%), respectively. The frequency of other HPV types was below 5%. HPV types 57, 64, 69 and 74 were not found in any of the tested samples by any assay. The overall typing capability of LA was the best (99.3%), followed by the BS-RLB assay (97.4%), LiPA (95.4%) and GP-RLB (94.8%) (Table 2).

By means of LA, LiPA, GP-RLB and BS-RLB, 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 in 55.6%, 37.3%, 43.1% and 52.9%. The majority of HPV multiple infections found were double infections: 58.8%, 54.4%, 66.7% and 60.5%, found by LA, LiPA, GP-RLB and BS-RLB assays, 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 1-8 HPV types/sample), the BS-RLB assay detected a mean of 2 HPV types per sample (range 1-6 HPV types/sample) and 1.7 HPV types per sample were identified by both LiPA and GP-RLB (range 1-6 HPV types/sample for LiPA and 1-4 for GP-RLB) (Table 3). Fischer's exact test showed that for assay common HPV types, LA and BS-RLB were the most sensitive in detecting multiple HPV infection (LiPA vs. LA, p=0.0015 and LiPA vs. BS-RLB, p=0.0067). GP-RLB detected fewer multiple infections than LA (p=0.0940) and BS-RLB (p=0.2320), but this was not significant in either case. There was no significant difference between the LA and BS-RLB assays (p=0.7206) or between LiPA and GP-RLB (p=0.1488).

Figure 1 compares the frequencies of HR and LR HPV types detected by the 4 assays. HPV types were listed in decreasing frequency of detection by any assay. Stars mark assay common HPV types for which Friedman's test shows a statistically significant discrepancy between the tested assays. 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 ($p=0.062$).

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

The strength of inter-assay agreement for each HPV type was calculated as an average of all inter-assay pair K 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 type 35 had the greatest average K value (0.94) and along with HPV types 11, 45, 51, 56, 58, 66, and 70 fits into a very good agreement group (K for these types ranging from 0.82 to 0.94). The inter-assay agreement for HPV types 16, 18, 31, 33, 39, 42 and 68 was good (K ranging from 0.61 to 0.79). HPV types 6,

52, 53, 59 and 73 had a moderate inter-assay agreement (K ranging from 0.54 to 0.58). The assays had a fair strength of inter-assay agreement for HPV type 54 (K=0.27). Inter-assay agreement for type 40 was worse than chance, having an average Kappa value of -0.002. For other HPV types the inter-assay agreement could not be determined.

In Table 4, the agreement between assays, on typing assay common types, in single *vs.* multiple infection cases is summarized. Thus, in 71.8% cases of single infection, all four assays agreed on the types present, while in cases of multiple infection, in only 6.2% cases was complete agreement found, while in 93.8% of samples of multiple infection, 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 based on assay common types. On the other hand, agreement was considered partial when any two or more assays agreed on one or more HPV types 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 prevalent HPV types.

All four assays, LA, LiPA, GP-RLB and BS-RLB, 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, LA and both RLB assays also provide the detection and typing of more LR HPV types than LiPA does (Table 1). This advantage of LA, GP- and BS-RLB can be interesting for epidemiological studies, i.e. some low abundant LR might be shown to be associated with neoplastic

255 changes in cervical squamous cells. For instance, in our study LR HPV type 70 as a
256 single infection was found unexpectedly often in HGL samples (data not shown); larger
257 epidemiological studies are necessary to possibly classify it among high risk HPV types.

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

262 The highest number of HPV types in a single sample was identified by the LA
263 assay followed by BS-RLB, LiPA and GP-RLB. This could indicate the low sensitivity of
264 LiPA and GP-RLB assays or an unspecific amplification and/or hybridization of LA and
265 BS-RLB assays. For assay common HPV types, LA and BS-RLB were the two most
266 sensitive assays in detecting multiple HPV infection (more than 1 HPV type in a sample),
267 while the least sensitive was the LiPA assay. However, we can hypothesize that these
268 data show LiPA as the most specific assay, while LA and BS-RLB reveal a certain level
269 of unspecific amplification and/or hybridization. This large difference in identified
270 multiple HPV infections could not be attributed to the fact that RLB and LA tested for
271 more HPV types than LiPA, as only assay common types were considered. This
272 observation was in disagreement with the results of Gillio-Tos et al. (12), who have
273 reported the LiPA assay as being more sensitive than the GP-RLB and Amplisense
274 assays.

275 The strength of inter-assay agreement for each assay common type was calculated
276 as an average K value of all assay pair combinations for that type (data not shown). It is
277 interesting to note that the most common HR HPV types (16, 18, 31 and 33) showed only
278 a good but not perfect strength of agreement. This observation suggests a possible

problem for accurate typing. Furthermore, significant differences were observed in 12 out of 22 HPV types common to all 4 assays. For HPV type 58, differences arose from the lower number of positive samples detected by the LA and LiPA assays when compared with both versions of the RLB assay, though 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 the least detected by GP-RLB and the most by LiPA, while HPV 56 was found the least by LiPA (Table 2, Figure 1).

HPV 45 was among the HPV types with very good agreement between assays, and no significant discrepancy was observed; it was detected only slightly less often by the LiPA assay.

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

Differences between assays for typing the most common LR HPV types 6 and 11 were not significant in either case, but the strengths in agreement between assays were very different for these types. Since our sample pool was artificially depleted for these types, the result is of limited value. In contrast, the highest strength of inter-assay

agreement was found for HPV types 35 and 66, indicating that these types were almost equally detected by all 4 assays.

Moderate agreement was also observed for types 52, 53, 59, and 73. This might pose a bigger problem, as all these types are HR 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 types HPV 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 GP-RLB having the lowest detection rate. We stress again that this overestimation is due to the LA assay design, and is a known limitation of this assay. Until this limitation is overcome, samples indicated to contain HPV 52 by LA assay should be viewed with greater caution in epidemiological studies. On the other hand, when evaluating clinical samples for diagnostic purposes, this probe cross-reactivity is not as important as the patient is already diagnosed with an HR HPV type (33, 35 or 58) and, regardless of HPV 52 positivity, should be considered as being at higher risk of the disease. The difference observed for HPV 53 had to be attributed primarily to the GP-RLB assay failing 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 the LiPA assay as in the case of HPV 68. However, when LiPA HPV 73 findings are compared with LA assay findings, LiPA shows a slight underestimation of this type's presence, while BS-RLB greatly underestimates it in comparison with LiPA or LA.

The only HPV type which showed a fair strength of inter-assay agreement was HPV 54. In this case, LiPA failed completely to detect it, while GP-RLB 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 LA and once by LiPA assays, but not in the concordant sample (Table 2). Even though the number of HPV 40 positive samples is very low this result suggests that the primer and probes should be re-evaluated in both RLB assays and/or the assays should be compared in 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 single infection cases, but so was complete disagreement. In multiple infection cases, partial agreement dominated and due to this, when all types of infections are combined, partial agreement is again the most common. This clearly indicates that LA, LiPA GP-RLB and BS-RLB assays do not in reality give the same answer when confronted with multiple infections, 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 GP-RLB, LiPA and Amplisense assays, and reported high assay concordance in single, but limited in multiple, infection cases. This is most probably due to the affinity differences of the primer sets for the different types. In addition, the concentration of a particular HPV type in the sample plays a role. In our experience, only the combination of typing methods with a knowledge of the limitation of each of them allows the unambiguous typing of HPV types, but the cost is unacceptable for routine settings.

When comparing similar studies to our results, both van Hamont et al. (36), and van Doorn et al. (35), comparing only LA and LiPA assays, have found these two assays to be highly comparable for assay common HPV types. While in our study, these assays showed a higher average K value than other assay pairs, we found their strength of

agreement to be good, though not very good. Contrary to our study, both of these studies were designed not to be challenging for the assays, analyzing usually normal cytology samples, a relatively small amount of multiple infections, and a normal distribution of HPV types. In our study, we wanted to evaluate them in more difficult conditions when low prevalent and/or multiple HPV types are present. In such a sample pool, there is a great possibility of different HPV types competing for the PCR reagents, and thus, especially when they are not present in the same amounts, PCR amplicons of one type might remain below the detection limit of the assay.

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

In this study, we have considered the ability of each of the four tested assays to detect a wide spectrum of less prevalent HPV types. We focused on inter-assay agreement, both overall and type-specific, in cases with single and/or multiple HPV infection. Our results showed a large variability in the ability of a particular assay to detect different HPV types. LA and BS-RLB found a higher rate of cases positive for multiple types than the two other assays. The lowest capability of detecting multiple infections was observed for the LiPA assay. Inter-assay agreement was moderate for

374 single infections and poor for multiple infections. The use of only one assay in
375 epidemiological and clinical studies might lead to biased conclusions. Therefore, a
376 universally evaluated and agreed upon HPV typing assay is needed for possible clinical
377 applications, or a combination of current assays, with a knowledge of their limitations, is
378 advised.

379

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518 **Table 1.** Genotyping capabilities of different assays used in this study

519 *According to Munoz et al.(22); ** LA assay cannot exclude HPV 52 in samples
520 containing either 33, 35 or 58; ***LiPA assay cannot distinguish types 68 and 73 from
521 each other

522

523 **Table 2.** Prevalence of HPV types present in samples as found by LA (Roche), LiPA
524 (Innogenetics), GP-RLB, BSP-RLB, any and all 4 assays

525 *LA cannot distinguish HPV 52 in samples containing either 33, 35 or 58; **LiPA
526 cannot distinguish HPV types 68/73; ND denotes HPV type undeterminable by the assay

527

528 **Table 3.** Types of HPV infections present in samples, identified by LA (Roche), LiPA
529 Innogenetics), GP-RLB, BS-RLB and common to all 4 assays.

530 *HPV types 6, 11, 16, 18, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70
531 and 73; **LA cannot distinguish HPV 52 in samples containing either 33, 35 or 58;
532 ***LiPA cannot distinguish HPV types 68/73

533

534 **Table 4.** Overview of the agreement between LA, LiPA, GP-RLB and BSP-RLB
535 regarding their capability of HPV type identification

536 *Complete agreement: identical HPV types detected with all 4 assays simultaneously in
537 one sample; partial agreement: one or more HPV types in common for two or more
538 assays; no agreement: different HPV types detected with all 4 assays **LA cannot
539 distinguish HPV 52 in samples containing either 33, 35 or 58 and LiPA cannot
540 distinguish HPV types 68/73

**Figure 1.** Detection frequencies of HR (A) and LR (B) HPV types by LA, LiPA GP-RLB and/or BS-RLB assays in 153 selected samples; HPV types are sorted by decreasing average frequency as detected with any assays; stars ✧ indicate significant differences (Friedman test) between assays for a particular HPV type; * indicates HPV types not included in all 4 assays (see Table 1); LA cannot distinguish HPV 52 in samples containing either 33, 35 or 58 and LiPA cannot distinguish HPV types 68 and 73 from each other

**Figure 2.** Average Kappa value of one by one inter-assay agreement measurements for each HPV type present in LA, LiPA, GP-RLB and BSP-RLB assays; * indicates HPV types for which significant differences were observed; LA cannot distinguish HPV 52 in samples containing either 33, 35 or 58; LiPA cannot distinguish HPV types 68/73

**Table 1.** Genotyping capabilities of different assays used in this study

| Oncogenic potential* | HPV type | LA (Roche) | LiPA (Innogenetics) | GP-RLB | BS-RLB |
|---|---|---|---|---|---|
| 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 | √** | √ | √ | √ |
| | 56 | √ | √ | √ | √ |
| | 58 | √ | √ | √ | √ |
| | 59 | √ | √ | √ | √ |
| | 68 | √ | √*** | √ | √ |
| | 73 | √ | √*** | √ | √ |
| | 82 | √ | | √ | √ |
| Probably HR | 26 | √ | | √ | √ |
| | 53 | √ | √ | √ | √ |
| | 66 | √ | √ | √ | √ |
| Number of detectable HPV types | | 37 | 26 | 37 | 37 |

*According to Munoz et al. (22); ** LA assay cannot exclude HPV 52 in samples containing either 33, 35 or 58; ***LiPA assay cannot distinguish types 68 and 73 from each other

**Table 2.** Prevalence of HPV types present in samples as found by LA (Roche), LiPA (Innogenetics), GP-RLB, BSP-RLB, any and all 4 assays

| HPV type | LA* | | LiPA** | | GP-RLB | | BS-RLB | | All 4 assays | | Any assay | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No | % | No | % | No | % | No | % | No | % | No | % |
| **No HPV** | 1 | 0.7 | 7 | 4.6 | 8 | 5.2 | 4 | 2.6 | 0 | 0.0 | 17 | 11.1 |
| **Any HPV** | 152 | 99.3 | 146 | 95.4 | 145 | 94.8 | 149 | 97.4 | 153 | 100 | 136 | 88.9 |
| **6** | 3 | 2.0 | 2 | 2.0 | 2 | 1.3 | 1 | 0.7 | 1 | 0.7 | 5 | 3.3 |
| **11** | 3 | 2.0 | 4 | 2.6 | 3 | 2.0 | 4 | 2.6 | 3 | 2.0 | 5 | 3.3 |
| **16** | 21 | 13.7 | 21 | 13.7 | 30 | 19.6 | 27 | 17.6 | 17 | 11.1 | 36 | 23.5 |
| **18** | 8 | 5.2 | 5 | 3.3 | 9 | 5.9 | 7 | 4.6 | 4 | 2.6 | 11 | 7.2 |
| **26** | 1 | 0.7 | ND | ND | 0 | 0.0 | 2 | 1.3 | ND | ND | 2 | 1.3 |
| **31** | 12 | 7.8 | 15 | 9.8 | 11 | 7.2 | 10 | 6.5 | 9 | 5.9 | 18 | 11.8 |
| **33** | 10 | 6.5 | 10 | 6.5 | 11 | 7.2 | 9 | 5.9 | 6 | 3.9 | 14 | 9.2 |
| **34** | ND | ND | 1 | 0.7 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 1 | 0.7 |
| **35** | 5 | 3.3 | 5 | 3.3 | 5 | 3.3 | 4 | 2.6 | 4 | 2.6 | 5 | 3.3 |
| **39** | 9 | 5.9 | 9 | 5.9 | 5 | 3.3 | 11 | 7.2 | 5 | 3.3 | 11 | 7.2 |
| **40** | 2 | 1.3 | 1 | 0.7 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 3 | 2.0 |
| **42** | 16 | 10.5 | 4 | 2.6 | 17 | 11.1 | 22 | 14.4 | 4 | 2.6 | 22 | 14.4 |
| **43** | ND | ND | 1 | 0.7 | 2 | 1.3 | 1 | 0.7 | 0 | 0.0 | 2 | 1.3 |
| **44** | ND | ND | 2 | 1.3 | 0 | 0.0 | 1 | 0.7 | 0 | 0.0 | 2 | 1.3 |
| **45** | 17 | 11.1 | 15 | 9.8 | 17 | 11.1 | 18 | 11.8 | 14 | 9.2 | 20 | 13.1 |
| **51** | 24 | 15.7 | 26 | 17.0 | 17 | 11.1 | 22 | 14.4 | 16 | 10.5 | 29 | 19.0 |
| **52** | 55 | 35.9 | 30 | 19.6 | 15 | 9.8 | 25 | 16.3 | 15 | 9.8 | 60 | 39.2 |
| **53** | 24 | 15.7 | 20 | 13.1 | 2 | 1.3 | 23 | 15.0 | 2 | 1.3 | 24 | 15.7 |
| **54** | 6 | 3.9 | 0 | 0.0 | 2 | 1.3 | 6 | 3.9 | 0 | 0.0 | 8 | 5.2 |
| **55** | 3 | 2.0 | ND | ND | 2 | 1.3 | 1 | 0.7 | 0 | 0.0 | 3 | 2.0 |
| **56** | 20 | 13.1 | 14 | 9.2 | 23 | 15.0 | 23 | 15.0 | 13 | 8.5 | 24 | 15.7 |
| **57** | ND | ND | ND | ND | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 |
| **58** | 24 | 15.7 | 25 | 16.3 | 32 | 20.9 | 33 | 21.6 | 21 | 13.7 | 36 | 23.5 |
| **59** | 8 | 5.2 | 4 | 2.6 | 2 | 1.3 | 5 | 3.3 | 2 | 1.3 | 10 | 6.5 |
| **61** | 12 | 7.8 | ND | ND | 0 | 0.0 | 7 | 4.6 | 0 | 0.0 | 12 | 7.8 |
| **62** | 5 | 3.3 | ND | ND | ND | ND | ND | ND | 0 | 0.0 | 5 | 3.3 |
| **64** | 0 | 0.0 | ND | ND | ND | ND | ND | ND | 0 | 0.0 | 0 | 0.0 |
| **66** | 17 | 11.1 | 19 | 12.4 | 16 | 10.5 | 16 | 10.5 | 15 | 9.8 | 19 | 12.4 |
| **67** | 2 | 1.3 | ND | ND | ND | ND | ND | ND | 0 | 0.0 | 2 | 1.3 |
| **68** | 5 | 3.3 | 12 | 7.8 | 4 | 2.6 | 5 | 3.3 | 4 | 2.6 | 12 | 7.8 |
| **69** | 0 | 0.0 | ND | ND | ND | ND | ND | ND | 0 | 0.0 | 0 | 0.0 |
| **70** | 8 | 5.2 | 8 | 5.2 | 8 | 5.2 | 9 | 5.9 | 7 | 4.6 | 9 | 5.9 |
| **71** | 0 | 0.0 | ND | ND | 1 | 0.7 | 1 | 0.7 | 0 | 0.0 | 1 | 0.7 |
| **72** | 1 | 0.7 | ND | ND | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 1 | 0.7 |
| **73** | 13 | 8.5 | 12 | 7.8 | 8 | 5.2 | 4 | 2.6 | 4 | 2.6 | 19 | 12.4 |
| **74** | ND | ND | 0 | 0.0 | ND | ND | ND | ND | 0 | 0.0 | 0 | 0.0 |
| **81** | 6 | 3.9 | ND | ND | 4 | 2.6 | 3 | 2.0 | 0 | 0.0 | 7 | 4.6 |
| **82** | 8 | 5.2 | ND | ND | 4 | 2.6 | 7 | 4.6 | 0 | 0.0 | 9 | 5.9 |
| **83** | 4 | 2.6 | ND | ND | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 4 | 2.6 |
| **84** | 15 | 9.8 | ND | ND | 0 | 0.0 | 2 | 1.3 | 0 | 0.0 | 15 | 9.8 |
| **IS39** | 1 | 0.7 | ND | ND | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 1 | 0.7 |
| **89** | 17 | 11.1 | ND | ND | 3 | 2.0 | 2 | 1.3 | 0 | 0.0 | 17 | 11.1 |

*LA cannot distinguish HPV 52 in samples containing either 33, 35 or 58; **LiPA cannot distinguish HPV types 68/73; ND denotes HPV type undeterminable by the assay

**Table 3.** Types of HPV infections present in samples, identified by LA (Roche), LiPA (Innogenetics), GP-RLB, BS-RLB and common to all 4 assays

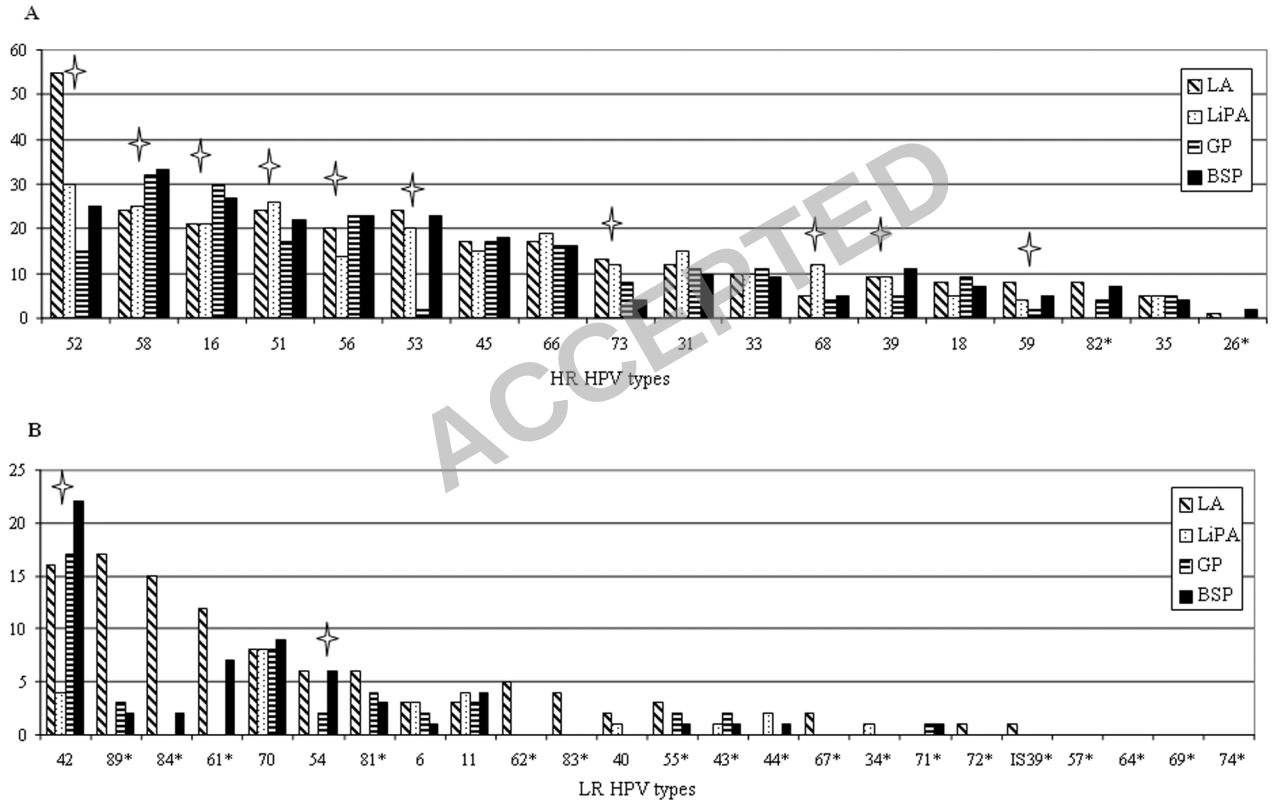
| HPV types | Type of infection | LA** | | LiPA*** | | GP-RLB | | BS-RLB | |
|---|---|---|---|---|---|---|---|---|---|
| | | No of cases | % | No of cases | % | No of cases | % | No of cases | |
| HPV types present in all 4 assays* | **Any infection** | 144 | 94.1 | 142 | 92.8 | 135 | 88.2 | 144 | 94 |
| | **Single** | 59 | 38.6 | 85 | 55.6 | 69 | 45.1 | 63 | 41 |
| | **Multiple** | 85 | 55.6 | 57 | 37.3 | 66 | 43.1 | 81 | 52 |
| | **2 types** | 50 | 32.7 | 31 | 20.3 | 44 | 28.8 | 49 | 32 |
| | **3 types** | 16 | 10.5 | 17 | 11.1 | 18 | 11.8 | 18 | 11 |
| | **4 types** | 11 | 7.2 | 7 | 4.6 | 4 | 2.6 | 10 | 6 |
| | **5 types** | 7 | 4.6 | 1 | 0.7 | 0 | 0 | 4 | 2 |
| | **6 types** | 1 | 0.7 | 1 | 0.7 | 0 | 0 | 0 | 0 |
| | **7 types** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | **8 types** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | **No infection** | 9 | 5.9 | 11 | 7.2 | 18 | 11.8 | 9 | 5.9 |

*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; **LA cannot distinguish HPV 52 in samples containing either 33, 35 or 58; ***LiPA cannot distinguish HPV types 68/73

**Table 4.** Overview of the inter-assay agreement between LA, LiPA, GP-RLB and BSP-RLB regarding their capability of HPV type identification

| Type of infection | Agreement* | HPV types present in all 4 assays** | |
|---|---|---|---|
| | | Number of samples (%) | Number of cases (% of group) |
| All infections | Complete | 152 (99.3) | 35 (23.0) |
| | Partial | | 111 (73.0) |
| | None | | 6 (3.9) |
| Single infections | Complete | 39 (25.5) | 28 (71.8) |
| | Partial | | 5 (12.8) |
| | None | | 6 (15.4) |
| Multiple infection | Complete | 113 (73.9) | 7 (6.2) |
| | Partial | | 106 (93.8) |
| | None | | 0 |
| Samples without HPV types present in all four assays | | 1 (0.7) | |

*Complete agreement: identical HPV types detected with all 4 assays simultaneously in one sample; partial agreement: one or more HPV types in common for two or more assays; no agreement: different HPV types detected with all 4 assays **LA cannot distinguish HPV 52 in samples containing either 33, 35 or 58 and LiPA cannot distinguish HPV types 68/73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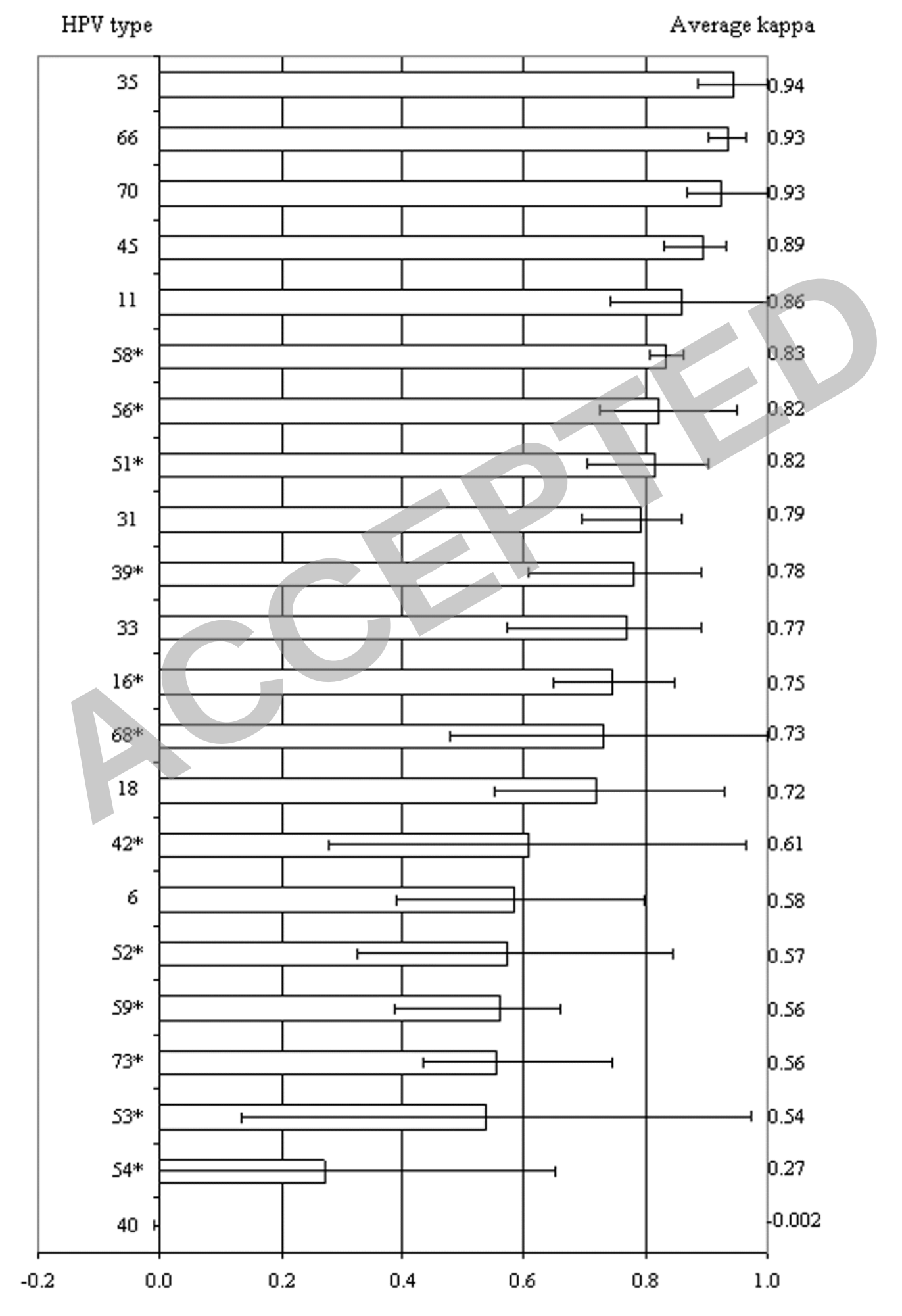
HPV type
Average kappa
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