CHAPTER 3

High genotyping concordance between the digene HPV
Genotyping RH Test and the Reverse Line Blot genotyping
assay on GP5+/6+ PCR products

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Journal of Clinical Virology 2009; S3: S16-S20
High genotyping concordance between digene RH Test and RLB


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Summary

Background: Based on epidemiologic studies, 18 mucosal human papillomavirus (HPV) types have been classified as (probably) high-risk (HR) (i.e., HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82). Recognition of HR HPV at the individual type level may be valuable in clinical management of HR HPV-positive women.

Objectives: The goal of this study was to evaluate the performance of the novel digene HPV Genotyping RH Test (digene RH Test), which uses type-specific probes for the 18 HR HPV genotypes, in comparison to the established in-house Reverse Line Blot (RLB) genotyping assay on PCR products generated with the clinically validated GP5+/6+-PCR method.

Study design: GP5+/6+ amplimers, generated from 493 digene High Risk HPV HC2 DNA Test (HC2)-positive and 95 HC2-negative cervical smears, were genotyped by both the digene RH Test and the RLB assay.

Results: Both genotyping assays demonstrated high concordance for overall HR HPV detection (κ = 0.886) and type-specific identification of the 18 HR types (overall κ = 0.951, individual κ range 0.777 to 1.000) in 493 HC2-positive samples. The digene RH Test revealed positivity for one or more HR HPV type(s) in 86.6% of the HC2-positive women, and negativity was confirmed in 97.9% of the HC2-negative women.

Conclusions: The digene HPV Genotyping RH Test revealed a high genotyping agreement with the established RLB assay on GP5+/6+ amplimers. Accordingly, this assay following GP5+/6+-PCR could serve as a follow-up test in a clinical setting for women who are HC2-positive to identify the respective HR HPV genotype(s).

Abbreviations

HPV: human papillomavirus
HR: high-risk
RLB: reverse line blot
PCR: polymerase chain reaction
HC2: Hybrid Capture 2
DNA: deoxyribonucleic acid
FDA: Food and Drug Administration
EIA: enzyme immunoassay
CIN: cervical intraepithelial neoplasia
UCM: Universal Collection Medium
RNA: ribonucleic acid
RLU/CO: relative light unit per cutoff value
LR: low-risk

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subsequently be subjected to the Reverse Line Blot (RLB) assay.\(^6\)

Recently, the Cervista HPV HR (detection of 14 HR types) and Cervista HPV 16/18 (genotyping of HPV 16 and 18) tests also received FDA approval.

Given the fact that certain HR HPV types, particularly HPV 16 and 18, confer an increased risk of cervical (pre)cancer,\(^5,6\) presence of these types might be an indication for more aggressive clinical management. Genotyping also allows monitoring the persistence of an HR HPV infection,\(^3\) which is linked to the risk of developing (pre)cancer.

The HC2 assay and GPS+6+PCR-EIA system performed equally well for the detection of cervical intraepithelial neoplasia grade 3 or cervical cancer (≥CIN3) in a population-based cervical screening well for the detection of cervical intraepithelial neoplasia grade 3 (pre)cancer.

\(^3\) The trial was approved by the national ethical committee (Ministry of Public Health) and all women enrolled had given informed consent. The first 493 HC2-positive samples encountered were as well as a random set of 95 HC2-negative samples were used in the current study.

2.3. DNA isolation

Of the left-over UCM specimens, DNA was isolated using PureK treatment followed by NucliSens EasyMAG automated nucleic acid extraction (bioMérieux, Bouxel, The Netherlands) according to the recommendations of the manufacturer.

2.4. GPS+6+PCR and Reverse Line Blot (RLB) analysis

The GPS+6+PCR was performed at the Department of Pathology, VU University Medical Center (Amsterdam, The Netherlands) as described previously.\(^6\) The resulting PCR products were split and half of the portions was used for RLB genotyping at the same department according to protocols that were described previously.\(^6\) The RLB assay used detects 37 HPV types (i.e., HPV6, 11, 16, 18, 26, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 61, 66, 68, 70, 71 [CP8061], 72, 73, 81 [CP8061], 83 [MM7], 84 [MM8], and CP6108). Of the left-over UCM specimens, DNA was isolated using ProtK extraction (bioM´erieux, Boxtel, The Netherlands) according to the kit manual in a ProfiBlot 48 analyzer (Tecan Austria GmbH, Salzburg, Austria). As an additional step, 10μl 38 buffer solution (Labo Bio-Medical Products BV, Rijswijk, the Netherlands) was added to the mix of GPS+6+PCR products and denaturation solution prior to hybridization to circumvent non-specific background. In short, subsequent hybridization with the strips occurred under stringent conditions followed by incubation with alkaline phosphatase–streptavidin conjugate. After addition of a substrate, reactive HR HPV probes were visually interpreted as a result of the enzymatic coloring reaction.

Testing with both the RLB assay and the digene RH Test was performed in parallel by different technicians who were unaware of each other’s test result and blinded to HC2 status of the samples.

2.5. digene HPV Genotyping RH Test (digene RH Test) analysis

The digene RH Test utilizes probes for 18 HR HPV types (i.e., HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82) that are similar to the respective RLB-probes, with minor modifications, and are immobilized on nitrocellulose strips. The test was performed on the other half of the generated GPS+6+PCR products at DDL Diagnostic Laboratory (Voorburg, The Netherlands) according to the kit manual in a ProfiBlot 48 analyzer (Tecan Austria GmbH, Salzburg, Austria). As an additional step, 10μl 38 buffer solution (Labo Bio-Medical Products BV, Rijswijk, the Netherlands) was added to the mix of GPS+6+PCR products and denaturation solution prior to hybridization to circumvent non-specific background. In short, subsequent hybridization with the strips occurred under stringent conditions followed by incubation with alkaline phosphatase–streptavidin conjugate. After addition of a substrate, reactive HR HPV probes were visually interpreted as a result of the enzymatic coloring reaction.

Testing with both the RLB assay and the digene RH Test was performed in parallel by different technicians who were unaware of each other’s test result and blinded to HC2 status of the samples.

2.6. Data analysis and statistics

For analytical comparison of the digene RH Test and the RLB assay for HR HPV detection, both methods were considered HR HPV-positive when genotyping revealed one or more of the 18 HR HPV genotypes that can be detected by both assays (i.e., HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82). Furthermore, for comparison to HC2 a distinction was made between HR HPV-positivity for the 13 HR HPV types targeted by HC2 (i.e., HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82) and the complete group of 18 HR HPV types targeted by the digene RH Test.

To investigate the agreement between both genotyping assays for one or more of the 18 HR HPV types, results were considered either as concordant (both methods yielded completely identical genotyping results), compatible (both methods showed one or more of the same genotype[s]), or discordant (no similarity between genotypes detected by both methods).\(^5\) Since the digene RH Test does not distinguish between IS939 and MM4 subtypes of HPV 82, positive RLB results for these subtypes were regarded as HPV 82-positive.

The two-tailed McNemar’s test was used for mutual comparison of the positivity rates of the RLB assay and the digene RH Test. The
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3. Results

3.1. Analytical performance of the digene RH Test

The specificity of the digene RH Test was evaluated with GP5+/6+ PCR products derived from a panel of cloned HPV types (i.e., HPV types 6, 11, 13, 16, 18, 26, 30, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 67, 68, 69, 70, 73, 74, 81, 82, 85, and 86). The digene RH Test clearly showed specific hybridization without any cross-hybridization to either closely related HPV types (Fig. 1) or non-targeted types (data not shown).

3.2. Comparison of the digene RH Test with the RLB assay for HRHPV detection

In order to compare the digene RH Test and the RLB assay for overall HRHPV detection, we analyzed GP5+/6+ PCR products derived from a series of 588 cervical smears previously testing positive (n = 493) and negative (n = 95) for HRHPV DNA by HC2. The same GP5+/6+ PCR products were used for the digene RH Test and the RLB assay. Results on the HC2-positives are shown in Table 1. The HRHPV detection rate by the digene RH Test (86.6%) was similar to that of the RLB assay (86.8%) in these HC2-positive specimens (k = 0.886). The 6 samples that were found HRHPV-positive only by the digene RH Test included one double infection with HPV 16 and 39, and 5 single infections comprising HPV 18, 31, 39 and 52 (twice), respectively. The RLB scored 7 samples additionally positive that included single infections of HPV genotypes 16, 35, 39, 56 and 59, respectively, and one mixed infection with the types 45, 52 and 58.

In the 95 HC2-negative cervical smears, the digene RH and RLB tests scored 93 samples as HRHPV-negative (97.9%), whereas 2 samples were identified as HRHPV-positive (i.e., having single infection with HPV 16 and 45, respectively) by both genotyping assays.

When restricting the analysis to the 13 HR HPV types detected by HC2, the digene RH Test scored 401 of the 493 (81.3%) HC2-positive cervical smears as HR HPV-positive. Twenty-six (5.3%) could be attributed to positivity for one or more of the five HR HPV types additionally targeted by the digene RH Test strip (i.e., HPV 26, 53, 66, 73 and 82). Seven samples (1.4%) in which the digene RH Test did not recognize any HPV, were HR HPV-positive according to the RLB assay, as discussed above. Amongst the 59 HC2-positive specimens (12.0%) that were HRHPV-negative by both assays, the RLB found 13/59 samples (22.0%) positive for LRHPV, including types HPV 6, 30, 67, and 70. In the remaining 46/59 samples (88.0%) no genotype was detected by both the digene RH Test and the RLB assay.

3.3. Comparison of HR HPV genotyping results of the digene RH Test versus the RLB assay

Table 1

<table>
<thead>
<tr>
<th></th>
<th>RLB</th>
<th>digene RH Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRHPV+</td>
<td>477</td>
<td>471</td>
</tr>
<tr>
<td>HRHPV−</td>
<td>62</td>
<td>59</td>
</tr>
</tbody>
</table>

k = 0.886 (95% CI: 0.825–0.947), P = 1.000.

HR HPV+ is defined as positive for HPV16, 18, 26, 31, 33, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and/or 82.

HR HPV− is defined as negative for HPV16, 18, 26, 31, 33, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and/or 82.

The individual genotyping findings among HC2-positives are shown in Table 2. The digene RH Test and RLB typing procedures showed an excellent overall agreement (k = 0.951) when all typing results of assay-common HPV types (i.e., HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82) were taken into account. Kappa values per individual HPV type ranged from 0.891 to 0.990.
Table 2
Comparison of genotyping findings between the digene RH Test and the RLB assay in 493 HC2-positive cervical smears

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of samples found positive by:</th>
<th>( \kappa ) (95% CI)</th>
<th>P-value*a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>digene RH Test and RLB</td>
<td>digene RH Test only</td>
<td>RLB only</td>
</tr>
<tr>
<td>HPV16</td>
<td>134</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>HPV18</td>
<td>32</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>HPV26</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HPV31</td>
<td>72</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>HPV33</td>
<td>18</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HPV35</td>
<td>9</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>HPV39</td>
<td>33</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>HPV45</td>
<td>28</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>HPV51</td>
<td>46</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>HPV52</td>
<td>49</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>HPV53</td>
<td>8</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>HPV56</td>
<td>28</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>HPV58</td>
<td>23</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>HPV59</td>
<td>9</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>HPV60</td>
<td>32</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>HPV68</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HPV73</td>
<td>6</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>HPV82</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Any typeb</td>
<td>536</td>
<td>32</td>
<td>20</td>
</tr>
</tbody>
</table>

*a McNemar’s test.

Table 3
The digene RH Test and the RLB assay in relation to the number of HR HPV types detected per specimena

<table>
<thead>
<tr>
<th>Test result, n (%)</th>
<th>digene RH Test</th>
<th>RLB</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of HR HPV types present within a sample</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>311 (72.8)</td>
<td>320 (74.8)</td>
<td>0.535</td>
</tr>
<tr>
<td>2</td>
<td>96 (22.5)</td>
<td>88 (20.5)</td>
<td>0.507</td>
</tr>
<tr>
<td>3</td>
<td>16 (3.8)</td>
<td>20 (4.7)</td>
<td>0.690</td>
</tr>
<tr>
<td>4</td>
<td>3 (0.7)</td>
<td>0 (0)</td>
<td>0.124</td>
</tr>
<tr>
<td>5</td>
<td>1 (0.2)</td>
<td>0 (0)</td>
<td>0.489</td>
</tr>
<tr>
<td>Total number of multiple infections (≥2 types)</td>
<td>156 (27.2)</td>
<td>108 (25.2)</td>
<td>0.535</td>
</tr>
</tbody>
</table>

*a Only genotypes commonly detected by both assays (i.e., HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82) are included.

Using this approach, the digene RH Test and the RLB assay demonstrated high concordance for the detection and genotyping of 18 HR HPV types in HC2-positive samples. The format of the home-brew RLB assay, which is currently used for genotyping of GP5+/6+-PCR-EIA-positive amplimers, is not easily implemented in routine laboratories. Consequently, the digene RH Test offers an easy-to-use alternative for the RLB assay.

4. Discussion

This study investigated the analytical performance of the digene RH Test for genotyping of GP5+/6+-PCR products, in comparison to the established RLB genotyping assay. The same series of GP5+/6+-amplimers generated from cervical specimens derived from a screening cohort were used. A comparison between two PCR-based assays on similar DNA input is influenced by differences in each consecutive step of the assay, including PCR primer design, choice of polymerase enzyme, and PCR protocol.11 By using the same GP5+/6+-amplimers in this study, variation in genotyping findings due to PCR differences was absent and the comparison could be focussed to the read-out of the amplimers.

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Lack of detection of HR HPV genotypes by the digene RH Test in 66 (13.4%) HC2-positive specimens could partially be explained by...
cross-reactivity of the HC2 assay with LR HPV types not included in the digene RH Test, but targeted by the RLB assay. These findings are in line with previous reports reporting cross-reactivity of the HC2 probe cocktail with untargeted types.12–14 Otherwise, it might reflect HC2 background noise using the standard cut-off for positivity (RLU/CO 1), supporting a threshold adjustment that has been reported earlier.12 Alternatively, insufficient amplification by the GP5+/6+ PCR, a step that the HC2 assay does not require, either or not influenced by inadequate DNA extraction or PCR inhibitors present in these samples, might have caused false-negativity by the PCR-based genotyping assays.

At the type-specific level, the few discrepant results were most likely due to low concentrations of target amplimers resulting in a test-to-test variation. Only the genotyping findings for HPV 16 differed statistically significantly (P < 0.05) between the RLB genotyping assay and the digene RH Test. This difference was not visible at the HR HPV detection level, as it mainly concerned increased recognition of HPV 16 by the digene RH Test in the context of multiple HR HPV infections as compared to the RLB assay. This may indicate the occurrence of competition between multiple genotypes, a problem common to all broad-spectrum PCR-based methods.15 If HPV 16 was the minor genotype and amplified into or not influenced by inadequate DNA extraction or PCR inhibitors present in these samples, the genotyping findings for HPV 16 remains to be determined, but may be of low consequence.

In conclusion, an algorithm combining the FDA-approved digene Hybrid Capture 2 High-Risk HPV DNA Test with the GP5+/6+ based digene HPV Genotyping RH Test offers promising possibilities in cervical cancer screening and triage.

Acknowledgements: The authors are grateful to Marja Kamp, Jacqueline Röer, Bianca Geze, Ilna van Haafsten (DDI Diagnostic Laboratory), Nathalie Fransen-Daalmeijer, Fatih Topal, and Dorien Rijkaart (Molecular Pathology unit, VU University Medical Center) for their technical assistance. Competing interests: CJLM is in the advisory board of QIAGEN. Funding: This study was supported by Stichting Pathologie Ontwikkeling en Onderzoek. The sponsor had no involvement in study design, data interpretation, writing of the manuscript and decision to submit the manuscript for publication.

Ethics approval: The current study followed the ethical guidelines of the Institutional Review Board of the VU University Medical Center.

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