CHAPTER 5

Universal human papillomavirus genotyping by the digene

HPV Genotyping RH and LQ Tests

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Chapter 5
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ABSTRACT

Background: High-risk (hr)HPV testing plays an important role in primary cervical cancer screening. Subsequent hrHPV genotyping might contribute to better risk stratification. The majority of hrHPV tests do not include identification of individual hrHPV genotypes.

Objectives: The digene HPV Genotyping RH Test (strip-based) and LQ Test (xMAP-based) allow genotyping of GP5+/6+ amplimers, but their probes target a region in the L1 ORF, which is also amplified by other broad-spectrum hrHPV assays, e.g., the Roche Amplicor HPV Test (Amplicor) and the Roche Linear Array. The goal was to test whether the RH Test and LQ Test can be used as an universal hrHPV genotyping test.

Study design: Self-collected cervico-vaginal specimens (n = 416) from an epidemiologic study were analyzed with Amplicor. The amplimers obtained were also tested with the RH Test and LQ Test for identification of 18 HPV types, including the 13 hrHPVs targeted by Amplicor.

Results: 197 specimens were positive by Amplicor, in which the RH Test and LQ Test identified one of the 13 hrHPVs in 94.4% and 98.0%, respectively. In 219 specimens remaining negative by Amplicor, the RH Test and LQ Test, performed on the Amplicor amplification products, still detected one of the 13 hrHPVs in 3.7% and 5.5%, respectively, and include identification of HPV53, 66, and 82. Overall, the RH and LQ Tests demonstrated high concordance with Amplicor for hrHPV detection (κ = 0.908 and κ = 0.923, respectively).

Conclusions: The digene HPV Genotyping RH and LQ Tests can be directly used for amplimers generated by the Amplicor HPV Test.

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Chapter 5

(strip- and xMAP-based) test formats utilize the same DNA oligo-probes that target a region in the L1 ORF, which is also covered by at least two other broad-spectrum HPV tests, e.g., the Roche Amplicor HPV Test and the Linear Array (PGMY-based test). The universal application of the RH Test and LQ Test for several L1-based amplification systems, e.g., Amplicor, PGMY, and GenoPlex, is important to investigate, since there is a lack of standardization in (hr)HPV genotyping.

2. Objectives

The RH and LQ Tests might be used for direct genotyping of Amplicor-amplified samples. The aim of this study was to investigate, since there is a lack of standardization in (hr)HPV genotyping.

3. Study design

3.1. Clinical specimens

A selection of 416 clinical specimens was obtained from a prospective epidemiologic study performed among unscreened women aged 18–29 years.16 Self-collected cervico-vaginal specimens, stored in 5 ml SurePath medium (Tripath Imaging, Burlington, NC, USA), had been analyzed by a HPV test algorithm, comprising amplification by SPF10 primers, detection by a DNA enzyme immunoassay (DEIA), and genotyping by SPF10 HPV LiPA, (strip- and xMAP-based) test formats utilize the same DNA oligo-probes that target a region in the L1 ORF, which is also covered by at least two other broad-spectrum HPV tests, e.g., the Roche Amplicor HPV Test and the Linear Array (PGMY-based test). The universal application of the RH Test and LQ Test for several L1-based amplification systems, e.g., Amplicor, PGMY, and GenoPlex, is important to investigate, since there is a lack of standardization in (hr)HPV genotyping.

3.2. DNA isolation

DNA was isolated from 500 μl of cervico-vaginal specimen on a MagNa Pure Robot (Roche Diagnostics, Almere, The Netherlands) using the MagNa Pure LC Total Nucleic Acid Isolation Large Volume Kit (Roche) and eluted in 50 μl of elution buffer. Isolated DNA was stored at −20 °C until further testing.

3.3. Amplicor HPV Test

HPV DNA was amplified using the Amplicor HPV Amplification Kit (Roche) according to the manufacturer’s instruction, with the deviation of using 10 μl of isolated DNA instead of 50 μl. Immediately after amplification, 50 μl of the PCR product was transferred to a new tube for further genotyping with digene RH and LQ Tests and stored at −20 °C to prevent residual Uracil-N-Glycosylase enzyme (UNG, AmpErase) activity. To the remaining 50 μl of Amplicor-generated amplimer, 50 μl Denaturation Solution (DN) was added. Simultaneous detection of 13 hrHPV types (i.e., HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) using 25 μl of denatured PCR product was carried out using the Amplicor HPV Detection Kit (Roche) according to the manufacturer’s instruction.

3.4. digene HPV Genotyping RH Test

The digene RH Test utilizes probes for 18 carcinogenic and borderline carcinogenic HPV types (i.e., HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68 (68a), 73, and R2 (IS39&MM4)). These probes are immobilized on colour-coded beads. The digene HPV Genotyping RH Test, Detection Kit was performed in the Luminex 100 IS System (Luminex Corporation, Austin, TX, USA) according to the manufacturer’s kit insert, using of 4 μl Ampliplier PCR product per hybridization. Thawed amplifiers were tested immediately to prevent degradation of PCR products by any residual UNG activity. A threshold for positivity of 100 MFI was applied.

3.5. digene HPV Genotyping LQ Test

The digene LQ Test uses probes for 18 carcinogenic and borderline carcinogenic HPV types (i.e., HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68 (68a), 73, and R2 (IS39&MM4)). These probes are immobilized on colour-coded beads. The digene HPV Genotyping LQ Test, Detection Kit was performed in the Luminex 100 IS System (Luminex Corporation, Austin, TX, USA) according to the manufacturer’s kit insert, using of 4 μl Ampliplier PCR product per hybridization. Thawed amplifiers were tested immediately to prevent degradation of PCR products by any residual UNG activity. A threshold for positivity of 100 MFI was applied.

3.6. Data analyses and statistics

To determine the agreement for hrHPV detection between the Amplicor HPV Test and the digene RH Test or LQ Test, the analysis was restricted to the 13 hrHPV genotypes that all three tests have in common (i.e., HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68). The two-tailed McNemar’s test was used for mutual comparison of positivity rates. The level of agreement was determined using Cohen’s kappa statistics. Level of statistical significance was set at 0.05. All analyses were performed using SPSS version 15.0.

4. Results

4.1. Roche Amplicor HPV amplification and detection

DNA from 416 self-collected cervico-vaginal specimens was analyzed by the Roche Amplicor HPV Test. Overall, 197 of 416 (47.6%) samples were found positive. In the remaining 219 (52.6%) samples no hrHPV was detected.

4.2. Genotyping of Roche Amplicor HPV-positive samples

The RH Test identified one or more of the 13 Ampliplayer-targeted hrHPV types in 186 (94.4%) of the 197 Amplicor-positive specimens. The LQ Test identified one or more of the 13 Ampliplayer-targeted hrHPV types in 193 (98.0%) of 197 specimens. The RH Test identified the 186 samples found positive by the RH Test. In 7 specimens, the LQ Test was positive, while the RH Test was negative, and these samples contained HPV16, 35 (n = 3), 58 (n = 2), and 59. Four Ampliplayer-positive samples could not be genotyped by the RH nor the LQ Test. In these four specimens, SPF10 LiPA had identified single hrHPV types 16 (n = 2) and 59 (n = 2).

The type distribution according to both genotyping assays among the 197 Amplicor-positive samples is shown in Table 1. HPV16, 51, and 52 are the most prevalent genotypes. Of the five HPV
Table 1

<table>
<thead>
<tr>
<th>Type</th>
<th>Number of samples found positive by</th>
<th>Kappa value (95% CI)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV16</td>
<td>54</td>
<td>0</td>
<td>0.914 (0.852–0.976)</td>
</tr>
<tr>
<td>HPV18</td>
<td>23</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td>HPV31</td>
<td>30</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td>HPV33</td>
<td>12</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td>HPV35</td>
<td>2</td>
<td>3</td>
<td>0.955 (0.125–1.000)</td>
</tr>
<tr>
<td>HPV51</td>
<td>21</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td>HPV45</td>
<td>9</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td>HPV47</td>
<td>34</td>
<td>1</td>
<td>0.962 (0.866–0.998)</td>
</tr>
<tr>
<td>HPV52</td>
<td>27</td>
<td>0</td>
<td>0.970 (0.958–1.000)</td>
</tr>
<tr>
<td>HPV56</td>
<td>21</td>
<td>0</td>
<td>0.903 (0.807–0.997)</td>
</tr>
<tr>
<td>HPV58</td>
<td>5</td>
<td>6</td>
<td>0.611 (0.329–0.849)</td>
</tr>
<tr>
<td>HPV59</td>
<td>5</td>
<td>2</td>
<td>0.828 (0.735–1.000)</td>
</tr>
<tr>
<td>HPV68</td>
<td>5</td>
<td>0</td>
<td>0.907 (0.735–1.000)</td>
</tr>
<tr>
<td>HPV69</td>
<td>0</td>
<td>0</td>
<td>0.995 (0.735–1.000)</td>
</tr>
<tr>
<td>HPV73</td>
<td>4</td>
<td>1</td>
<td>0.916 (0.612–1.000)</td>
</tr>
<tr>
<td>HPV16/18</td>
<td>3</td>
<td>1</td>
<td>0.974 (0.938–1.000)</td>
</tr>
<tr>
<td>HPV66</td>
<td>7</td>
<td>1</td>
<td>1.000</td>
</tr>
<tr>
<td>HPV33/39</td>
<td>4</td>
<td>1</td>
<td>0.906 (0.807–0.997)</td>
</tr>
<tr>
<td>Any type</td>
<td>256</td>
<td>23</td>
<td>0.936 (0.914–0.958)</td>
</tr>
</tbody>
</table>

* Not targeted by Amplicor Detection Kit.
* McNemar’s Test.

4.4. Overall HPV detection by Amplicor, RH Test and LQ Test

In the total group of 416 specimens, Amplicor hrHPV-positive (n = 197) and negative results (n = 219) were largely confirmed by the RH Test (κ = 0.908; Table 2). The LQ Test also demonstrated excellent concordance with Amplicor for hrHPV detection in the complete study panel (κ = 0.923; Table 2). Discrepancies between the RH Test and Amplicor were observed in 19 (4.6%) of 416 specimens. The LQ Test and Amplicor results were discordant in 16 (3.8%) specimens. No statistically significant difference was observed between assay results (P = 0.648 for RH Test vs Amplicor and P = 0.077 for LQ test vs Amplicor). The latter P value, being close to the level of statistical significance (0.05), implies a non-significant trend for a difference between the LQ Test and Amplicor.

5. Discussion

Different hrHPV genotypes demonstrate a variation in risk for the development of cervical cancer.4–6 Therefore, genotyping after general hrHPV-based testing might contribute to better clinical management.13 Genotyping following hrHPV detection allows monitoring of viral persistence and determining the risk of an individual woman for the development of cervical precancer.

This study demonstrated that the digene HPV Genotyping RH Test (strip-based) and LQ Test (Luminex-based) permit direct genotyping by analysis of amplifiers that were generated by the Amplicor HPV Test. This allows both assays to be implemented in a test algorithm as genotyping assays of Amplicor hrHPV-positive cervical specimens.

The RH Test and LQ Test probes are compatible with three widely used broad-spectrum PCR primer sets in the L1 ORF, i.e., Amplicor, GP5+/6+14,15 and PGMY (based on pilot study results, data not presented). This creates the possibility of ‘universal’ genotyping after Amplicor- or GP5+/6+-based hrHPV detection assays by RH Test and LQ Test. The RH Test or LQ Test eliminate the need for separate genotyping assays and could therefore contribute to standardization in hrHPV testing.

Roche recently launched the Cobas 4800 HPV Test system, allowing detection of 14 hrHPV types and genotyping of HPV 16
Overall, the RH Test and LQ Test both demonstrated very good concordance with Amplicor (κ = 0.908 and 0.923, respectively) for ‘detection’ of 13 hrHPVs. The LQ Test revealed slightly more genotypes than the RH Test (P < 0.001), the majority in multiple infections. Apparently, the LQ Test platform is more sensitive.

When compared with Amplicor, previous SPF10 LiPA hrHPV detection results (for 13 hrHPVs) confirmed 193/197 Amplicor-positive and 154/219 Amplicor-negative specimens. Four samples were positive by Amplicor alone, and 65 specimens only by SPF10 LiPA (data not presented in this study). Although SPF10 LiPA confirmed the presence of hrHPV in virtually all Amplicor-positive specimens, its higher positivity rate was not consistent with an earlier report by Van Ham et al., describing an almost complete similarity. In that study, clinical materials comprised cervical scrape specimens from women attending a gynaecologist. In that study cohort, hrHPV-positive scapes probably contained a relatively high viral load, resulting in an equivalent detection rate by both assays. In contrast, the current study panel consisted of self-collected cervico-vaginal swab specimens from young, unscreened women. This population generally has a low viral load and high clearance rate, explaining the higher positivity rate by the analytically more sensitive SPF10 LiPA method. This finding is similar to a previous case-control comparison study. In the control group, SPF10 LiPA demonstrated a higher positivity rate than the SPF5+6+ method, whereas hrHPV detection rate of both assays was equal in the case group. Real-time PCR determined HPV16, 18 and S2 type-specific viral loads, which were significantly lower in the GPS5+6+ negative but SPF10 LiPA-positive group compared to the GPS5+6+ positive and SPF10 LiPA-positive group. This illustrates that differences in viral load can explain differences between assays.

In conclusion, this study has demonstrated that the RH Test and the LQ Test can be used in a novel test algorithm as direct genotyping assays. The Amplicor-positive PCR products. In addition, these genotyping assays demonstrated that type-specificity of the Amplicor HPV Test is determined at the probe detection level, and not at the amplification level.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>digestion RH Test</th>
<th></th>
<th>digestion LQ Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrHPV+</td>
<td>186</td>
<td>11</td>
<td>101</td>
</tr>
<tr>
<td>hrHPV−</td>
<td>8</td>
<td>211</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>(x = 0.008 (95% CI 0.868–0.940), P = 0.048)</td>
<td></td>
<td>(x = 0.921 (95% CI 0.868–0.960), P = 0.007)</td>
</tr>
</tbody>
</table>

* McNemar’s Test.

**SUPPLEMENTARY TABLE 2**

Comparison between the Amplicor HPV Test and the digene RH Test and LQ Test for the detection of hrHPV in a selection of 416 clinical specimens.

<table>
<thead>
<tr>
<th></th>
<th>Amplicor</th>
<th></th>
<th>Amplicor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td><strong>HR Test</strong></td>
<td>186</td>
<td>11</td>
<td>101</td>
</tr>
<tr>
<td><strong>LQ Test</strong></td>
<td>8</td>
<td>211</td>
<td>12</td>
</tr>
<tr>
<td><strong>Digene</strong></td>
<td>(x = 0.008 (95% CI 0.868–0.940), P = 0.048)</td>
<td></td>
<td>(x = 0.921 (95% CI 0.868–0.960), P = 0.007)</td>
</tr>
</tbody>
</table>

Chapter 5

and 18 in a single reaction (IPV Conference 2010). This novel system was designed to have an improved clinical specificity compared to the Amplicor HPV Test. Several studies are ongoing to evaluate the clinical performance of this novel assay.

In order to investigate the compatibility of the RH Test and LQ Test with Amplicor amplimers, self-collected cervico-vaginal specimens were available from a prospective epidemiologic study. Material from self-sampling techniques appears to be highly representative for the cervical HPV status.

In the current study, four specimens positive by Amplicor detection (and previously also positive by SPF10 LiPA) could not be genotyped by RH or LQ Tests. This might be due to a low amount of amplimer present in the sample causing test-to-test variation. It could also be possible that the genotyping probes of the RH and LQ Test target a different part of the Amplicor amplimer than the Amplicor detection probes. If a sequence variation was present in the region targeted by the RH/LQ Test probes only, this could explain the positivity found by only SPF10 LiPA and Amplicor. Unfortunately, no information regarding the Amplicor probe sequences was available to verify this possibility. Sequence analysis on these samples could confirm mismatches with the genotyping probes. Alternatively, since no Amplicor Denaturation Solution (DN) was added to the amplimers prior to performance of the RH and LQ Tests, residual UNG activity (which is active below temperatures of 55°C) might have degraded the PCR products. The DN was not added, since this would have diluted the already low amount of amplimers required as input for the RH Test and LQ Test (i.e., 10 μl and 4 μl, respectively). In addition, both assay protocols contain their own denaturation steps, and, although not investigated, the DN could have interfered with the tests.

The Amplicor assay comprises an amplification and a detection step. The detection is limited to the detection of 13 hrHPV types, but more genotypes may be amplified during the amplification step of the assay. This was shown by genotyping specimens with a negative Amplicor read-out using the RH and LQ Tests. Within this group, hrHPV-negativity was demonstrated in 211 (RH Test) and 207 (LQ Test) of the 219 samples. However, additional HPV types 53, 66, and 82 (not targeted by the Amplicor detection step) were identified in 17 (RH Test) and 18 (LQ Test) Amplicor-negative specimens, mostly as single genotype. In most cases, presence of HPV53 and 66 was confirmed by SPF10 LiPA (whereas HPV82 is not targeted by this test). These findings indicate that the Amplicor Amplification kit amplifies at least three additional types, which remain undetected by the Amplicor Detection Kit.

However, 8 and 12 of 219 Amplicor-negative samples were shown to contain one of the 13 hrHPVs by RH Test and LQ Test, respectively. These discrepant results are probably due to presence of suboptimal amounts of Amplicor amplimers. Small variations in amplimer input could have resulted in positivity for genotyping but negativity for Amplicor detection. Retesting the amplimers on the Amplicor platform might generate a value above the threshold for positivity. The identification of hrHPV types in Amplicor-negative specimens is only of academic value, as the envisioned algorithm comprises genotyping of Amplicor hrHPV-positive samples only.
References