DETECTION AND TYPING OF HUMAN PAPILLOMAVIRUSES BY PCR

E. Shikova¹, I. Todorova¹, G. Ganchev², V. Kouseva-Dragneva³
¹Bulgarian academy of sciences, Institute of Experimental Pathology and Parasitology, Sofia, Bulgaria
²National Specialized Hospital for Active Treatment in Oncology, Sofia, Bulgaria
³Sofia Dermatovenereological Dispensary, Sofia, Bulgaria
Correspondence to: Evelina Shikova

ABSTRACT

Human papillomavirus (HPV) infection is the leading risk factor for cervical intraepithelial neoplasia (CIN) and cervical cancer. Detection of high-risk HPV infections might identify women who are at increased risk of development or progression of a cervical lesion and may have prognostic significance. Therefore there is considerable interest in identifying and accurately typing the viruses. PCR amplification of HPV genomes is the most sensitive method for the detection of cervicovaginal HPV. The different PCR techniques used to detect HPV infection, however, affect the frequency with which viral DNA is identified. Using different PCR primer sets, we tried to optimize the detection and typing of HPVs. We tested both consensus and type-specific primers. PCR utilizing the two most commonly used consensus primer sets, MY09/MY11 and GP5+/GP6+, located within the L1 region of HPV genome, amplified a broad spectrum of HPV genotypes in a single reaction. To identify the HPV types, samples were also subjected to PCR using specific primers for HPV types 6/11, 16, 18, 31 and 33. Our results suggest that testing with both PCR using L1 consensus primers MY09/MY11 and with PCR using type-specific primers could provide the maximum accuracy of HPV identification.

Keywords: consensus primers, genotyping, human papillomavirus (HPV), PCR

Introduction

The association between cervical cancer and human papillomavirus (HPV) is well established (7, 16). More than 100 HPV genotypes are identified, but several of them, including HPV 16, 18, 31 and 33 are members of the high risk (HR) HPV group as they promote carcinogenesis. HPV type 16 is the most oncogenic type, followed by type 18.

Since persistence of oncogenic HPV types is important risk factor and predictor of cervical intraepithelial neoplasia (CIN) progression and cancer, identification of oncogenic HPV types has prognostic significance. Therefore there is considerable interest in detection and typing of these viruses. Many molecular methods for HPV testing are currently available but PCR amplification of HPV genomes is the most sensitive technique and can detect between 5 and 100 DNA molecules in a specimen. PCR utilizing consensus primers, directed at relatively conserved regions of the HPV genome, allows the amplification of a broad spectrum of HPV genotypes in a single reaction. A number of different primer combinations amplifying fragments from various regions of the HPV genome have been developed (14). The most frequently used amplification systems for the detection of HPV DNA in clinical samples are based on MY09/MY11 and GP5+/GP6+ consensus primer sets mediated PCR, amplifying DNA fragments in the conserved L1 region of approximately 450bp and 150bp respectively. Further, type-specific PCR primer sets allow the identification of individual genotypes.

Several studies indicate that any single method or technique for the detection of HPV may underestimate the true prevalence of HPV in cervical samples (4, 11). Since detection of HR HPV infections is crucial for identifying women who are at increased risk of development or progression of a cervical lesion, optimization of methods for HPV testing is an important task.

In order to optimize the PCR-based assays, used for HPV identification, we compared the ability of different primer sets to detect and type HPV DNA in clinical materials. We evaluated several consensus and type-specific (TS) PCR assays as tools to determine the HPV types in cervical specimens.

Materials and methods
Clinical materials
In this study, 54 cervical scrapes with normal and abnormal cytomorphology were analysed. The samples with cytological abnormalities were from women with low- and high-grade lesions of the cervix. The samples of cervical cells were taken using a cytobrush and placed in 1 ml 0.9% NaCl.

Sample processing
The samples were vigorously stirred and centrifuged at 4,000 x g for 10 min at room temperature. The cell pellet was resuspended in 500 μl of detergent buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 0.45% Triton X100, 0.45% Tween 20) and digested with 100 μg/ml proteinase K (Promega) for 2 h at 56°C (12). The proteinase was inactivated by incubation at 95°C for 10 minutes. The processed samples were stored at -20°C antil analysis was performed. 10μl of processed samples was used in each PCR assay.

PCR amplifications
Each sample was subjected to 9 paralell PCR reactions using the following primer sets: consensus primers MY09/MY11 and GP5+/GP6+ and type-specific primers for HPV6, 11, 16 (16L1 and 16E6/7), 18, 31 and 33 (1, 12, 13). All primers used in this study are shown in Table 1.

The final 50μl PCR mixture contained 10-μl sample, 25 μl PCR Master Mix (Promega), 3 mM MgCl2, 20 pmol of each primer. Amplifications were performed with the following cycling profile: incubation at 94°C for 5 min followed by 40 cycles of 1-min denaturation at 95°C, 1-min annealing at 55°C, and 1-min elongation at 72°C. The last cycle was followed by a final extension of 10 min at 72°C. The primer annealing step of GP5+/GP6+ primers-based PCR was performed at 40°C for 2 min.

To check the DNA quality the specimens were amplified with beta-globin primers PC04 and GH20. During amplification positive and negative control samples were included. As positive controls CaSki cell DNA (HPV16 positive) and HeLa cell DNA (HPV18 positive) were used.

PCR products were analysed on a 2% agarose gel stained with ethidium bromide and visualized by UV-transillumination.

<table>
<thead>
<tr>
<th>Primers used in this study</th>
<th>Primers</th>
<th>Sequence (5'-3')</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consensus primers</td>
<td>MY09</td>
<td>CGTCC(AC)A(AG)(AG)GGA(T)ACTGATC</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>MY11</td>
<td>GC(AC)CAGGG(AT)CATAA(C)TAAATGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GP5+</td>
<td>TTTGTTACTGTGGTAGATGATACGCTAC</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>GP6+</td>
<td>GAAAAATAAAACTGTAATCATATT</td>
<td></td>
</tr>
<tr>
<td>Type-specific primers</td>
<td>6.1</td>
<td>TAGTGGGCTATGGCTGTCA</td>
<td>280</td>
</tr>
<tr>
<td></td>
<td>6.2</td>
<td>TCCATTAGCCTCCAGGGGTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11.1</td>
<td>GGAATACATTGCGCCATGGG</td>
<td>360</td>
</tr>
<tr>
<td></td>
<td>11.2</td>
<td>CGGCCGAGCTCGGTCTCTCG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16.L1-1</td>
<td>TGCTAGTGCTATGCGACAAA</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>16.L1-2</td>
<td>ATTTACTGCAACATTGTCAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16.E6-7-1</td>
<td>TGGCTTTTTCGGGATTTATGC</td>
<td>390</td>
</tr>
<tr>
<td></td>
<td>16.E6-7-2</td>
<td>AGATCGAGTTGTCTCTTGGTGCA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18.1</td>
<td>AAGGATGCTGACCCCGCCTGA</td>
<td>216</td>
</tr>
<tr>
<td></td>
<td>18.2</td>
<td>CACGCACACGCTTGCGGCAGTTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>31.1</td>
<td>ATGGTGATGTCACAAAAACACC</td>
<td>514</td>
</tr>
<tr>
<td></td>
<td>31.2</td>
<td>GTGATGCTACAGGACAACTGAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>33.1</td>
<td>ATGATGATGATGTTGACGCC</td>
<td>455</td>
</tr>
<tr>
<td></td>
<td>33.2</td>
<td>GCACACTCCATGCGATCAG</td>
<td></td>
</tr>
</tbody>
</table>

Results and Discussion
We examined 54 cervical specimens obtained from cytologically normal women and from women with low- and high-grade lesions of cervix for the presence of HPV DNA by PCR using consensus and type-specific primers to determine if HPV detection varies when different primer sets are used. First we have compared the two major consensus primer sets used for PCR amplification of HPV DNA, MY09/MY11 and GP5+/GP6+ primers. The MY09/M11
primers are synthesized with several degenerate nucleotides in each primer and are thus a mixture of 25 primers, capable of amplifying a wide range of HPV types (6). The GP5+/GP6+ primers consist of fixed nucleotide sequence for each primer and detect a wide range of HPV types by using a lowered annealing temperature during PCR (1). The MY09/MY11 primer set has been used predominantly in studies in America and Asia, whereas the GP5+/GP6+ primer system has been used primarily in Europe (9). Fragments of 450 bp and 150 bp are amplified by PCR using MY09/MY11 and GP5+/GP6+ primers, respectively (Fig. 1). In our study both, MY09/MY11 and GP5+/GP6+ consensus primer sets, exhibited approximately equal sensitivity, as defined by the ability to detect HPV DNA. 15 (27.8%) and 14 (25.9%) of samples were HPV positive in PCR using MY09/MY11 primers and GP5+/GP6+ primers, respectively. GP5+/GP6+ mediated PCR failed to detect HPV DNA in 1 sample found HPV positive by MY09/11 PCR. All HPV DNA negative samples showed positivity in beta-globin gene PCR analysis.

Fig. 1. HPV detection by consensus primers PCR: 1. PCR with MY09/MY11 primers; 2. PCR with GP5+/GP6+ primers; 3. Molecular weight marker (100 bp)

HPV typing is of great importance for cervical cancer risk assessment, as well as planning individual treatment and vaccination strategies (3, 10). We tested cervical specimens by PCR using type-specific primers for the most common HPV types - HPV6, 11, 16, 18, 31 and 33 (6, 12, 13). The most prevalent was HPV16, detected with an L1 gene-specific primer pair in 5 (9.2%) samples, followed by HPV31 – in 3 (5.6%) samples, HPV6 – in 2 (3.7%) samples and HPV33 and HPV11 – in 1 sample each (1.85% each). We were unable to detect HPV18 DNA in analyzed samples. Three of samples, found HPV positive by consensus primers, were untipped with type-specific primers. The relatively high rate of untipped samples in this study indicates that PCR using primer sets for more HPV types should be included in the future analysis of cervical specimens.

Using TS PCR with primers directed to the L1 gene we found that HPV16 is the most common type. In addition, HPV16 is the main risk factor for cervical cancer. At the same time HPV type 16 is often found integrated into the chromosomes of cervical cells that disrupts the integrity of the E1 and E2 genes, while the E6 and E7 genes are retained. Therefore, we tried to optimize the TS PCR-based detection of HPV16 by using primers that amplify 390 bp fragment in E6/7 region of the HPV genome (Fig. 2). With this primer set we detected HPV16 DNA in 2 more samples in addition to the samples found HPV16 positive with L1 primer set. They were both negative by PCR with MY09/MY11 and GP5+/GP6+ consensus primers and by HPV16 PCR using L1 type-specific primers. These results showed that the E6/7 gene directed TS PCR increased the positivity rate for HPV16 DNA detection. Similar results are reported by other authors (2, 4, 5). The lower HPV16 positivity rate found with TS PCR based on L1-gene specific primers could be explained with partial deletion of the L1 region during the integration of HPV 16 into the host DNA. The HPV integration followed by deletion of virus genome could occur not only in the late phases of the neoplasia but is also found in some of low-grade lesions indicating that it may be an early event in cancer progression (8). In addition, a recent study showed that the frequency of integrated HR HPV genomes is with marked differences for individual HR HPV types (15) with HPV16, 18, and 45 found substantially more often in the integrated state compared with HPV types 31 and 33.

In conclusion, primer configurations used in HPV DNA amplification are important factor that determine sensitivity and specificity of PCR-based assays. Our results indicate that complementary PCR techniques based on both consensus and type-specific primers should be used to avoid missing
potential HR HPV infections. An appropriate HPV diagnostic system could include preselection of samples with the MY09/MY11 primers, followed by HPV typing by TS PCR with primers from E6-E7 regions of HPV genome.

Acknowledgment
This work was supported by Bulgarian National Science Fund, Grand No 1513-05.

REFERENCES