Detection and Typing of Human Papillomavirus in Cutaneous Common Warts by Multiplex Polymerase Chain Reaction

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A number of epidemiological studies have identified human papillomavirus (HPV) types 1, 2, 3, 4, 7, 10, 27, 57, and 65 in cutaneous common warts. However, identification of the HPV subtype by conventional polymerase chain reaction (PCR) is time consuming with its multi-step laboratory process. In this study, we aim to develop a specific one-step multiplex polymerase chain reaction method which can identify six different HPV genotypes related to common warts. By HPV DNA sequence analysis, 6 pairs of specific primers were designed from the intergenic regions of genes L1 to E6, and from genes E2 to L2. DNA sequence analysis with the L1 gene sequence of the sample was performed to measure the specificity of multiplex PCR. HPV-1, -2, -3, -4, -7, and -57 were identified without cross amplification in 109 out of 129 samples. The sensitivity and specificity of our set of primers in detecting HPV were 85% and 99.5%, respectively. For the 20 samples where HPV type was not identifiable by our batch of primer sets, multiplex PCR with an additional set of HPV primers was done, where 7 were found positive for HPV-7 or -65. Our results demonstrate that the newly designed multiplex PCR can rapidly detect the specific HPV subtype involved in common warts with high accuracy.

Key words: Common wart, human papillomavirus (HPV), multiplex-PCR, PCR, wart

Introduction

Human papillomaviruses (HPVs) are small double-stranded DNA viruses with a genome size of approximately 8000 base pairs that infect epithelial cells causing benign proliferation of skin warts or malignant mucosal tumors [2,6]. From DNA sequence analysis [20], HPV is known to comprise a significantly heterogeneous family of more than 130 types [1,5]. The HPVs have been classified into cutaneous, mucosal and epidermodysplasia verruciformis (EV) types according to their location and clinical context [4]. 'High-risk' HPV types have been implicated in the development of intraepithelial lesions and cervical cancer [17]. The high-risk types are HPV-16, -18, -31, -33, and -45, while the low-risk types are HPV-6 and HPV-11 [14]. Epidemiological studies also revealed that cutaneous HPV types such as HPV-1,-2,-3,-4,-7, -10, -27, -57, and -65 cause common warts on the skin (verrucae vulgaris) [10], and prevail dominantly in the European population [11] as well as Chinese [13]. They are often limited to the hands or the feet sparing other body sites.

The overwhelming number of HPV genotypes identified during the past decade has posed a challenge to the development of a simplified HPV DNA detection kit. The polymerase chain reaction (PCR) is proved to be the most sensitive method for identifying the presence of HPV infection in clinical samples [16]. The HPV genotype can be determined by analyzing the PCR product. However, the sequence diversity among various HPV types limits the number of viruses that can be detected by a single PCR primer set. Among the primer combinations that can amplify DNA fragments from various regions of the HPV genome, MY09/MY11 and GP5/GP6 for the conserved capsid protein L1 region have been most widely used in clinical practice, allowing detection of a broad range of mucosal HPV types [15,19].

In this study, we aimed to develop a specific one-step multiplex PCR method that can identify different HP types closely related to cutaneous common warts. The multiplex PCR with specific primers were performed in the 129 wart tissue samples.

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Materials and Methods

Clinical samples
A total of 129 tissue specimens were obtained from 109 patients with cutaneous warts on the hands and feet. Seoul St. Mary's Hospital and Chung-Ang University Yongsan Hospital participated in this study. Peeling followed by cryo-therapy with liquid nitrogen is the routine treatment protocol for cutaneous common warts. The warty tissue was obtained by the peeling process. An informed consent was obtained from all patients who participated in the study.

Primer design
The HPV genome is composed of six early (E1, E2, E3, E4, E6, and E7) and two late (L1 and L2) proteins. Aligned sequences from the intergenic region between E2 to L2 and L1 to 3' end of the genomes in the Genbank Database were manually compared to identify regions with the least degree of nucleotide sequence match. The nucleotide sequences of the six specific primers are described in Table 1. The HPV type 57-specific primer contains a single Y (T or C) base in its sequence. Primers MY09 (5'-GTGAGGAGTGAAGATTTTG-3') and MY11 (5'-GTCGAGGAGTGAAGATTTTG-3') were used as degenerate PCR primers for HPV typing [15]. To demonstrate that DNA samples are positive templates for the PCR, primers PC03 (5'-ACACAAGCTGTGTCACGTACCC-3') and PC04 (5'-CTCCAGGCGTCAACTGGGGC-3') were used to amplify the endogenous β-globin region of human genome.

Preparation of sample DNA
The frozen tissues were digested with lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 10 mM EDTA, pH 7.8, 2% SDS and 200 μg/ml of Proteinase K) at 37°C overnight. After removing protein debris twice by phenol-chloroform extraction, the DNA was precipitated with ethanol and re-suspended in water.

Multiplex PCR
The PCR was performed over a solution with a total volume of 30 μl, containing six primer pairs (10 μM each), DNA extract from a frozen sample, and a 1× Premix solution (Solgent, Korea). 30 cycles of amplification was carried out with each cycle running in the following parameter; denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 1 min.

Results
Design of multiplex PCR primers
HPV-1, -2, -3, -4, -6, -7, -27, -57 and -66 are well known to cause common warts. Especially, HPV-2, -27, and -57 are closely related and associated with skin warts. Among them, we chose common warts-related 6 HPV subtypes for identification by the multiplex PCR [3,9]. To set up a multiplex PCR for rapid identification of HPV-1, -2, -3, -4, -27, and -57, type-specific primers were designed from the intergenic region of each viral genome (Fig. 1). By comparing the HPV DNA sequences, we found that most variable regions were located between E2 and L2 open reading frame (ORF) or L1 and 3' end of the genome. Based on the aligned sequences, pairs of primers with a length of 20 bp and without homology were designed, amplifying distinguishable sizes of 575, 222, 348, 437, 174 and 511 bp, respectively (Table 1).

Table 1. Multiplex PCR primers used in this study

<table>
<thead>
<tr>
<th>HPV</th>
<th>Primers</th>
<th>Sequences (5'-3')</th>
<th>Region</th>
<th>BP</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Forward</td>
<td>tctatttggaggctcttcagttactatga</td>
<td>L1-3' end</td>
<td>575</td>
<td>7101-7676</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>aacgactacaattacaatgtaa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Forward</td>
<td>atacatttcagcatgtaaatgctagctagc</td>
<td>E2-L2</td>
<td>222</td>
<td>3891-4113</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>cagctagtgtgtaactgtaactagcagctga</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Forward</td>
<td>tctatttggaggctcttcagttactatga</td>
<td>E2-L2</td>
<td>348</td>
<td>3907-4255</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>aacgactacaattacaatgtaa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Forward</td>
<td>tctatttggaggctcttcagttactatga</td>
<td>L1-3' end</td>
<td>437</td>
<td>6909-7246</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>aacgactacaattacaatgtaa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>Forward</td>
<td>tctatttggaggctcttcagttactatga</td>
<td>E2-L2</td>
<td>174</td>
<td>4091-4264</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>aacgactacaattacaatgtaa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>Forward</td>
<td>gtcgactacaattacaatgtaa</td>
<td>E2-L2</td>
<td>511</td>
<td>3843-4354</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>aacgactacaattacaatgtaa</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HPV: Human papilloma virus; BP: base pairs
Preparation of the standard HPV type DNA

With HPV-1, -2, -3, -4, -27 and -57 type-specific primers, specific sequences flanking the target region were obtained. The HPV types were confirmed by DNA sequencing and Blast DNA database analysis. Each DNA from the 6 positive PCR products was then cloned into pHINT-T vector and the resultant recombinant plasmids were used as standard HPV type DNA [12]. PCRs of standard HPVs were performed using its specific primer to test whether DNA of the expected size was produced. From PCR amplification, the bands of 575, 222, 348, 437, 174 and 511 bp of HPV-1, -2, -3, -4, -27 and -57 type were detected, respectively (Fig. 2).

Genotyping of HPVs of common warts by multiplex PCR amplification

All HPV-1, -2, -3, -4, -27 and -57 types of different sizes were amplified simultaneously from the combined 6 standard HPV DNA mixed with their primer sets (Fig. 3, lane 1). The difference in size of the amplification products on agarose gel allowed rapid and specific discrimination between HPV type 1, 2, 3, 4, 27 and 57.

After confirming that the multiplex amplification works properly, a total of 129 tissue specimens of common warts from Korean patients were subjected to multiplex PCR. Figure 3 shows some results. By comparing the size of DNA on 2% agarose gel by standard multiplex PCR, we were able to identify their genotypes. The testing of 129 samples by multiplex PCR showed that 85% could be identified by their HPV types. The prevalence of HPV-1, 2, 3, 4, 27 and 57 in skin warts was 93%, 32.6%, 15%, 7%, 17.8%, and 13.2%, respectively (Table 2). Additional positive warts detected by the multiplex PCR system comprised of 4 samples co-infected with HPV types 2 and 27, or 2 and 57 (data not shown). All samples negative with multiplex PCR primers were positive for β-globin amplification.

Specificity and sensitivity

During PCR, there can be an experimental risk of cross-contamination between the reactions, leading to mis-typing of the wart samples. To make sure the HPV typing results are correct, the 109 HPV positive samples were amplified with the degenerate primers corresponding to the L1 ORF region. DNA sequencing and BLAST analysis of L1 PCR DNA fragment showed that HPV types determined by multiplex PCR were 99% correct, indicating high specificity of the specific primer sets in multiplex PCR (Table 3). Among the HPV types causing cutaneous warts, type 2, 27 and 57 are closely related and show more DNA homology to each other. In HPV identified cases, the specificity of the primer pairs for HPV-2, -27, or -57 type was analyzed by 3 independent PCR. As illustrated in Fig. 4, each PCR primer

Table 2. Distribution of HPV types identified by multiplex PCR in common warts samples

<table>
<thead>
<tr>
<th>Type</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>7</th>
<th>27</th>
<th>57</th>
<th>65</th>
<th>Mixed</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (%)</td>
<td>12 (9.3)</td>
<td>42 (32.6)</td>
<td>2 (1.5)</td>
<td>9 (7)</td>
<td>3 (2.3)</td>
<td>23 (17.8)</td>
<td>17 (13.2)</td>
<td>4 (3.1)</td>
<td>4 (3.1)</td>
<td>13</td>
<td>129</td>
</tr>
</tbody>
</table>

HPV: Human papilloma virus
Specific HPV has conserved high (85%) sequences served to generate homologies. HPV typing was confirmed by DNA sequencing of each L1 region of positive samples.

<table>
<thead>
<tr>
<th>Detection</th>
<th>Specific primer set (multiplex)</th>
<th>MY09/11 primers (consensus)</th>
<th>Confirmed by sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>109/129* (85%)</td>
<td>34/129 (27%)</td>
<td>108/109* (99%)</td>
</tr>
<tr>
<td>Negative</td>
<td>20/129 (15%)</td>
<td>95/129 (73%)</td>
<td></td>
</tr>
</tbody>
</table>

* Total sample number
b Positive sample number of six HPV types

HIV typing was confirmed by DNA sequencing of each L1 region of positive samples.

Table 3. Comparison of sensitivity and specificity between PCR with multiplex primer and PCR with MY09/11 consensus primer

Fig. 3. Multiplex PCR with the wart samples. Lane 1: multiplex PCR with mixed standard HPV-1, -2, -3, -4, -27 and -57 DNAs; lane 2: sample 1; lane 3: sample 2; lane 4: sample 3; lane 5: sample 4; lane 6: sample 5; lane 7: sample 6. Numbers denoted under the bands indicates HPV type.

was specific to the corresponding HPV type because there was no cross reaction with the other two sets of primer. The result demonstrates that the type-specific primer set can discriminate even closely related HPV types with high DNA homologies.

To evaluate the sensitivity of the multiplex PCR, the degenerate primer pairs MY09/11 were applied in PCR reaction of the same 129 samples tested with multiplex PCR. Out of 129 wart samples, PCR amplified bands were observed in 39 cases (26.4%) with MY09/11 primers, while the multiplex PCR products were detected from 109 samples (85%) with 99% specificity (Table 3). This clearly shows that our specific primer sets designed for multiplex PCR have high specificity and sensitivity.

Discussion

PCR with primers of matching nucleotide identity and conserved sequences in the L1 and E1 open reading frame has been successfully used to detect a wide range of genital HPV types in mucosal warts or epidermodysplasia verruciformis [7,19]. However, the identification of HPV types with degenerated primers require nested PCR, RFLP, or hybridization. In addition, compared to the large number of molecular epidemiological studies performed on HPV infection in genital warts, HPV infections in common warts have not been widely analyzed.

In this study, we present a specific one-step multiplex PCR method which permits identification of 6 different HPV genotypes related to common warts. Using a computer-mediated HPV DNA sequence analysis, 6 pairs of specific primers were designed from the intergenic regions between genes L1 and 3'-end of genome, or between genes E2 and L2. The detection of each HPV type depends on the identification of 6 amplification products of different sizes on agarose gel following multiplex PCR. We ran the multiplex PCR with HPV DNA isolated from 129 samples of common warts. Out of the HPV positive 109 samples, HPV-1, -2, -3, -4, -27, and -57 were identified without any cross amplification. The specificity of HPV-positive samples by the multiplex PCR assay was confirmed by DNA sequence anal-
ysis with the L1 gene sequence. The clinical specificity of the primer sets was 85%, which was significantly higher than those from PCR with the consensus sequence primers MY09/11 [8]. In this study, the most frequently observed HPV type in common warts was HPV type 2. HPV-27, -57 and -1 were also prevalent, showing similar results with the German [11] and Chinese study [13]. However, Hagiwara et al. [9] reported that the most frequent HPV types found in common warts among Japanese patient were HPV-1, 4, and 65. For the 20 samples with unidentified HPV, primers for HPV-7, 10 and 65 were added for trial. Of the 20 cases, 3 were positive to HPV type 7, and 4 to HPV type 65 (Table 2). Since the remaining samples negative with multiplex PCR were positive with β-globin PCR, these seem to contain other types of HPV. Therefore, we feel that there is a need to add a number of primers for different HPV types.

In conclusion, our data demonstrate that multiplex PCR with specific primers is a rapid and efficient method to identify the HPV types involved in common warts. The tissue samples required for the multiplex PCR is safe and easy to obtain; we can simply use the peeled-off products preceding cryotherapy. We hope our multiplex-PCR method encourages many to participate in studies with cutaneous common warts, of which the importance has been under-estimated despite its prevalence in the past.

Acknowledgements

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References

초록 : Multiplex PCR 기법을 이용한 보통사마귀 내 인유두종바이러스 검출 및 분류

최순용 2 · 임종호 3 · 김은정 1 · 김혜성 1 · 김범준 3 · 강훈 1 · 박영민 1 *

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현재까지 다수의 역학연구를 통해 피부에 발생한 보통사마귀에서 제 1, 2, 3, 4, 7, 10, 27, 57 및 65형의 인유두종바이러스가 검출되었다. 그러나 기존의 중합효소연쇄반응 (conventional polymerase chain reaction, PCR)을 이용하는 경우 절차가 복잡하여 시간이 오래 걸리는 단점이 있었다. 이번 연구를 통해 저자들은 보통사마귀에서 가장 흔히 검출되는 6가지 유전자형의 인유두종바이러스를 한 번에 확인 가능한 간편한 multiplex PCR의 개발을 목표로 하였다. 인유두종바이러스의 염기서열분석을 통해, L1에서 E6, 그리고 E2에서 L2 사이의 유전자간영역 (intergenic region)으로부터 6쌍의 primer를 고안하였으며, L1 유전자간영역 분석을 통해 multiplex PCR의 특이성을 확인하였다. 총 129개의 조직표본 중 109개에서 제 1, 2, 3, 4, 7, 27형의 인유두종바이러스를 확인하였다. 이번 연구의 primer를 이용한 인유두종바이러스 검출의 민감도와 특이도는 각각 85%와 99.5%였다. 이러한 primer 세트로 인유두종바이러스가 검출되지 않은 20개의 조직표본의 경우, 또 다른 HPV primer를 사용한 추가적인 multiplex PCR을 시행하여 7개 표본에서 제 7형 및 65형의 인유두종바이러스가 검출되었다. 이와 같은 결과는 본 연구를 통해 새롭게 고안된 multiplex PCR 기법을 통해 보통사마귀에서의 인유두종바이러스를 보다 정확하고 빠르게 검출할 수 있다는 것을 보여 준다.