The Double Polymerase Chain Reaction with Consensus Primers Permits Rapid and Sensitive Detection of Genital Human Papillomavirus Oncogenes

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ABSTRACT

We have developed a sensitive procedure for the detection of relatively low copy numbers of multiple genital human papillomaviruses (HPVs) using the polymerase chain reaction (PCR). HPV DNAs were detected by agarose gel electrophoresis and ethidium bromide staining after 2 rounds of PCR amplification (double PCR) with outer and inner consensus primer pairs for HPV-6, 11, 16, 18, 31, 33, 52, and 58. The detection limit of this method (i. e., 10^{-2} copy of HPV DNA per cell in 1 μg cell DNA) was sufficient for analysis of cervical intraepithelial neoplasia (CIN) specimens. Overall prevalence rate of HPV was 100% in 20 cases of CIN specimens. HPV typing by restriction enzyme analysis revealed that HPV-16 sequence was present in 11 cases, HPV-18 in 1 case, HPV-31 in 4 cases, HPV-33 in 1 case, HPV-52 in 2 cases, HPV-58 in 3 cases, and an unidentified type(s) in 3 cases. There were 4 cases of mixed infections. This procedure obviates the use of hybridization-based format for identification of at least 8 types of HPV sequences present in a small fraction of cells within a heterogeneous population.

Key words: Polymerase chain reaction, PCR, Nested PCR, Human papillomavirus, HPV

INTRODUCTION

The close association of human papillomaviruses (HPVs) with cervical can-

cer suggests a role of this virus in the development of cervical neoplasia. In particular, HPV-16, 18, 31 (1), 33 (2), 52 (3, 4), 58 (5), and others (6) are commonly or occasionally found in malignant anogenital lesions, whereas HPV-6 and -11 occasionally in benign cervical hyperplasias (6). Efficient detection and accurate typing of these different HPV genotypes in normal and diseased tissue samples have proven important prognostic indicators and to be useful for clinical diagnosis. Polymerase chain reaction (PCR) using consensus primers has been applied to detect a broad spectrum of HPV genotypes. The elaborated primers have sequences homologous to regions of the E1 (7, 8) and L1 (8-11) open reading frames (ORFs). In cervical carcinoma, the genome of integrated HPV is often deleted but the long control region (LCR), and the E6 and E7 ORFs, the transforming region of the HPVs, are conserved (12-16).

Recently, we have developed a novel PCR method to detect a broad spectrum of HPV genotypes using consensus primers, located in the E6/E7 region (17). However, this system has a limit of detection of approximately 0.1 copies/cell in 1 μg cell DNA, possibly due to mismatches of sequence between the primers and templates. The sensitivity of this method may be insufficient for the detection of HPVs from clinically normal cervical tissues, cervical intraepithelial neoplasia (CIN) tissues, and cervical smears. Thus, improved methodologies for higher HPV detection sensitivity will be necessary for valid epidemiological studies. A two-step amplification, first with outer primers and then with inner primers nested within the first primers, increases the specificity and sensitivity of the PCR. Greater sensitivity is also obtained by a combination of consensus PCR and Southern blot hybridization analysis. Although PCR-Southern blot technique offers a tremendous increase in the ability to detect HPV DNA, it is time-consuming and utilizes a radiolabeled probe that must be prepared frequently.

In this paper, a double-consensus PCR consisting of two-step amplifications with newly designed consensus outer sense primer for HPV-6, 11, 16, 18, 31, 33, 52, 58 and former inner primer sets was developed and applied to 20 cases of CIN specimens. We show that HPV DNA can be detected in 100% from the CIN specimens by the double PCR and simple visualization in agarose gels.

METHODS

DNA preparation from tissue specimens

Tissue biopsy samples were frozen immediately with liquid nitrogen and stored at −70℃. DNA was prepared by a standard proteinase K/SDS/phenol procedure as described (18). One μg of cellular DNA was used in the amplification reaction.

Oligonucleotide primers

Oligonucleotide primers were synthesized on a DNA synthesizer (Milligene 7500) and purified on an Oligonucleotide Purification Cartridge (Applied Biosystems). The nucleotide sequences of the primers and their locations in the HPV-16 genome are shown in Fig. 1.

PCR

The double-PCR was performed in a two-step reaction, first with a pair of outer primers and then with a pair of inner (i. e., nested) primers.

First PCR: PCR amplification was performed on 1 μg of cellular DNA or various amounts of cloned HPV plasmid DNA with 1 μg of cellular DNA from normal human cervical tissues in 100 μl of a reaction solution containing 10 mM Tris HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM each dATP, dTTP, dCTP, dGTP, 100 pmol each outer primers (pU-O/pU-2R) and 2.5 U of *taq* DNA polymerase (Perkin Elmer Cetus). Thirty cycles of PCR were carried out, consisting of denaturation at 94℃ for 1 min, annealing at 55℃ for 2 min, and extension at 72℃ for 3 min.

Second PCR: One μl of the first PCR product was submitted to amplification with the inner primers (pU-1M/pU-2R). PCR was carried out in 20 μl containing 20 pmol of each inner primer, the other reagents described above and 0.5 U of *taq* DNA polymerase. The PCR product was analyzed after thirty cycles of amplifications: denaturation at 94℃ for 1 min, annealing at 50℃ for 2 min, extension at 72℃ for 2 min.

To avoid false positives, " no template DNA " reagent control was included with each set of amplification and special care was taken to avoid contamination, as described previously (19). One-tenth (10 μl) of each PCR product was electrophoresed on composite gels consisting of 3% NuSieve agarose (FMC Bioproducts) and 1% Seakem agarose (FMC Bioproducts) to obtain adequate resolution of low-molecular-weight DNA fragments, then stained with ethidium bromide and photographed under UV light.

HPV typing

The HPV types were determined by restriction enzyme digestion as described (17). An aliquot of PCR products (nine-tenth) were purified by phenol-chloroform-isoamyl alcohol (25 : 24 : 1, vol/vol) extraction, followed by ethanol precipitation, and were suspended in 34 μl H_2O. One-fourth of them were digested with 3~4 units of *Ava* II, *Rsa* I, *Bgl* II, or *Acc* I in 10 μl reaction mixture for 2 hrs at 37℃. Digestion products were analyzed on the composite gels described above.

RESULTS

Primer design

Outer primer pU-O was chosen from the highly conserved region among HPV-6, 11, 18, 31, 33, 52, 58 to yield PCR products from a wide range of genital HPVs. Fig. 1 shows the location of pU-O primer on HPV-16 genome, which is located in the LCR (long control region). LCR, E6 and E7 regions are invariably present in cervical carcinomas containing HPV DNA (12-16). The viral sequences corresponding to pU-O of 8 types HPV are shown in Fig. 2

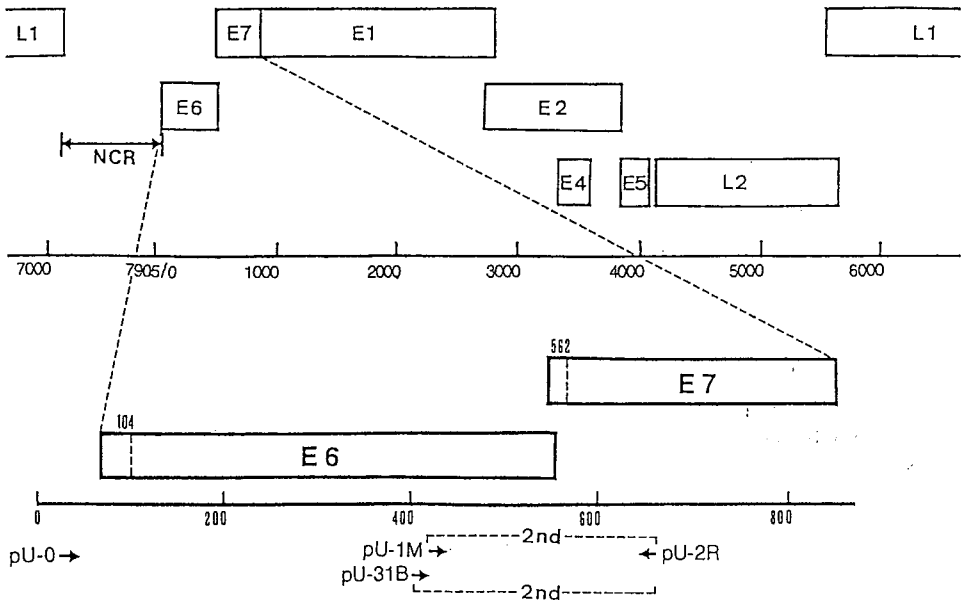

Fig. 1 Location and sequence of pU-1M, pU-2R primers (17) and pU-O primer in the HPV-16 genome. Arrowheads represent the 3' end of each primer.

	5' 3'	5'Nucleotide	Mismatches
Sense primer pU-0	AGGGAGTGACCGAAAACGGT		
HPV-6	TA----G----------	27	3
HPV-11	GA----G----------	27	3
HPV-16	----C--A-------T----	27	3
HPV-18	-------A----------	34	1
HPV-31	---------------GT---	30	2
HPV-33	----T--A-------G----	31	3
HPV-52	----T--A----------	31	2
HPV-58	----T--A----------	32	2

Fig. 2 Alignment of pU-O with corresponding sequences of several HPV types. The symbol represents identical bases; mismatched bases are indicated. The 5' nucleotide position of the sequences and the number of mismatches are given.

Increased detection limits with nested PCR methods

We previously showed that the detection limit of HPV DNA was 10^{-1} copy per cell by our single PCR with pU-1M/pU-2R (17). Preamplification of the HPV DNA with the first PCR primer set increased the sensitivity of this system (Fig. 3). Samples containing 10^{-2} copies of pHPV-16 per cell produced a clearly visible DNA band of expected size (i. e. 238 bp). Thus, the sensitivity of the double PCR was at least 10 fold higher than Southern blot hybridization and the pU-1M/pU-2R single PCR. The sensitivity to clonal HPV-6, 11, 18, 31, 33, 52, and 58 DNAs was approximately the same as that to clonal HPV-16 DNA (data not shown).

HPV detection in clinical samples

DNA samples that were analyzed by pU-1M/pU-2R single PCR were analyzed again by the double PCR. Samples were prepared as described in Methods, then 1 μg of DNA (10^5 cells) was used for each amplification reaction (single PCR) and for the first step amplification (double PCR). After double PCR, the products were purified and digested with restriction enzymes for type determination as described in ref. 17. As shown in Fig. 4, double PCR gave very clear products without a nonspecific background. PCR product from mild dysplasia 155 produced a cleavage pattern of HPV-52, and those from severe dysplasia 188 contained both of the HPV-16 and 31 patterns by double digestion with *Ava*II/*Rsa*I. CIS 4 and CIS 205 showed the cleavage patterns of HPV-16.

The results of 20 CIN samples analyzed by single and double PCR are summarized in Table 1. By the single PCR, 12 cases out of 20 (60%) contained high-risk HPV sequences. The double PCR was able to amplify the HPV sequences from 8 cases, all of which were judged negative for HPV by the single PCR. Furthermore, mixed infections were detected by double PCR from 2 cases

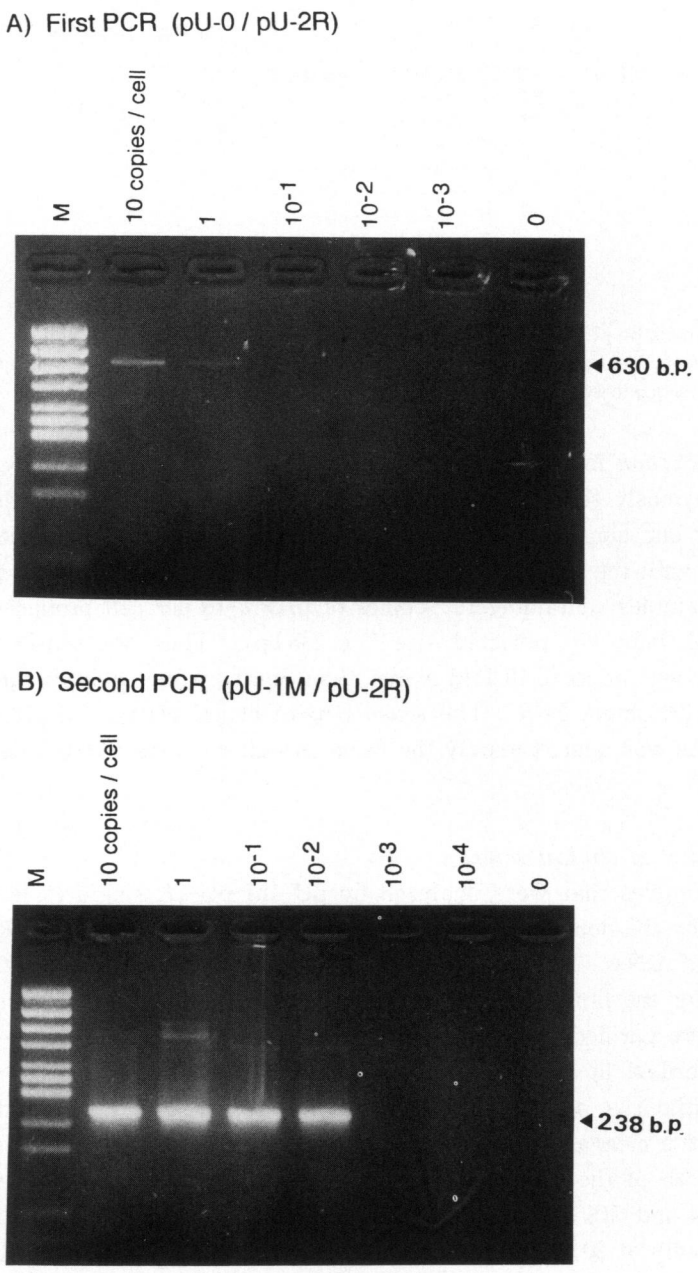

Fig. 3 Sensitivity of the double PCR. 10-fold serial diluted plasmid HPV-16 DNA was mixed with 1 μg HPV-negative human cervical DNA and used as a template. Lane M; DNA molecular weight marker, *Hae* III digested ϕX 174.

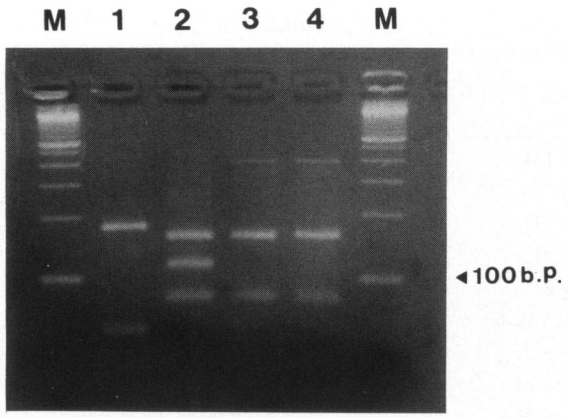

Fig. 4 Typing of double PCR products from CIN samples. 1 μg of DNA from CIN samples were subjected to 30 cycles of amplification with pU-0/pU-2R primer set; this was followed by 30 cycles of amplification with pU-1M/pU-2R primer set.
Lane M; DNA molecular weight marker, 100 bp ladder (GIBCO BRL).
Lane 1; mild dysplasia 155, *Bgl* II digestion
Lane 2; severe dysplasia 188, *Ava*II/*Rsa*I digestion
Lane 3; CIS 4, *Ava* II digestion
Lane 4; CIS 205, *Ava* II digestion

(mild dysplasia 213 and severe dysplasia 188) which were indicated to be single type positive by single PCR. We detected unidentified type(s) from three cases by the double PCR. These three cases showed different restriction patterns from each other.

DISCUSSION

We have designed two pairs of consensus primers in the E6/E7 region which can amplify at least 8 types of genital HPV DNA before (17), but the sensitivity of this system was similar to that of the Southernblot method, 0.1 copies/cell in 1 μg DNA. When dealing with a low-copy-number nucleic acid template, nested (double) PCR is a reliable way of improving sensitivity and specificity (20). The process utilizes two consecutive PCRs. The first PCR contains an external pair of primers, while the second contains one of the first primers and a single nested primer. The larger fragment produced by the first PCR is used as a template for the second PCR. Using double PCR, we have successfully improved the sensitivity by at least 10-fold.

We compared the detectability of HPV types in clinical samples between the single PCR and the double PCR. With the double PCR, false negative cases determined by the single PCR were all positive. Furthermore, mixed infection

Table 1

DNA source	HPV type	
	Single PCR[1]	Double PCR[2]
Mild dysplasia 154	58	58
Mild dysplasia 155	none	52
Mild dysplasia 194	31	31
Mild dysplasia 210	none	Unknown
Mild dysplasia 213	16	16, 58
Moderate dysplasia 68	none	Unknown
Severe dysplasia 27	16	16
Severe dysplasia 85	16	16
Severe dysplasia 164	none	18, 58
Severe dysplasia 188	16	16, 31
Severe dysplasia 215	16	16
Severe dysplasia 223	31	31
CIS 4	none	16
CIS 69	none	unknown
CIS 86	16	16
CIS 135	16	16
CIS 143	16	16
CIS 161	16	16
CIS 187	none	31, 33, 52b
CIS 205	none	16
	12/20	20/20

[1] One step amplification of pU-1M/pU-2R (ref. 17).

[2] Two step amplification of pU-O/pU-2R and pU-1M/pU-2R.

was found from 4 cases which had been found by the single PCR contain no or only one type of HPV. These results suggest that double PCR is a useful method to detect HPV DNA with sufficient high sensitivity. This nonradioactive PCR system is simple and convenient for clinical use. By double PCR, we detected the unidentified type(s) of HPV from cases in which single PCR had failed. New types of HPV could be detected because of the high sensitivity of the double PCR. The clinical implications of specific HPV types in cervical carcinomas should be appreciated. Our results indicate that the incidence of each HPV type varied with the severity of clinical samples. Mild dysplasia and severe dysplasia have various HPV types, while the incidence of HPV-16 seems to increase with the progression from severe dysplasia to carcinoma *in situ*. These results suggest that HPV-16-containing lesions are at high-risk, and the efficient detection and typing of HPV is useful for a prognostic indicator.

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