

DETECTION OF HUMAN PAPILLOMA VIRUS IN
PARAFFIN-EMBEDDED TISSUE USING THE POLYMERASE
CHAIN REACTION

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Human papilloma virus (HPV) infection of the cervix has been associated with neoplastic change (1). HPV can be classified into over 40 different types, based on hybridization criteria. Only certain types are commonly found in the female genital tract, with HPV 6 and HPV 11 associated with condyloma accuminatum and HPV 16 and HPV 18 associated with cervical dysplasia and carcinoma. The presence of HPV 16 or HPV 18 appears to be a major risk factor for progression of dysplasia to carcinoma. Current methods of HPV detection are cumbersome. No practical culture system for viral propagation is available. Antibodies against HPV antigens are generally not type specific and cannot detect HPV antigens in dysplastic or neoplastic cells since these antigens are poorly expressed in undifferentiated cells (2). DNA hybridization probes are required for specific viral typing. The DNA probes are usually hybridized against cervical biopsy DNA on a Southern blot, though dot blot type assays on DNA from cervical swabs have been described (3). In situ DNA hybridization can detect HPV but suffers from lack of sensitivity in dysplastic cervical lesions as the amount of viral DNA is reduced with increasing dysplasia. An in situ RNA probe has been able to detect and type HPV in cervical squamous cell carcinoma, but requires 4 wk of autoradiography (4).

To facilitate the understanding of HPV infection and its role in carcinogenesis, we sought to improve on current methods of detection. Ideally one would assay readily available clinical samples with high sensitivity and specificity for HPV type. We chose to adapt the new in vitro gene amplification technology, the polymerase chain reaction (PCR) for this purpose (5, 6). In the PCR, target DNA sequences are selectively amplified through repeated cycles of denaturation, annealing with oligomer primers complementary to flanking regions of the target sequence, and primer extension with DNA polymerase I (Klenow fragment). The amount of target DNA increases exponentially as a function of the number of cycles, facilitating subsequent detection with a labeled complementary oligomer. Since paraffin-embedded specimens are easily obtainable, the ability to study this material would be of great value. DNA can be extracted from paraffin-

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embedded tissue (7, 8) and subjected to restriction endonuclease analysis. In addition, portions of the human beta globin gene have been amplified using the PCR on DNA extracted from paraffin-embedded tissue (9). We adapted the PCR to paraffin-embedded tissue for the detection of HPV. Instead of extracting the DNA before amplification, we were able to directly use a single thin slice of embedded tissue as the amplification target, thereby saving considerable time and precisely defining the lesion under investigation. We chose to detect HPV 16 and HPV 18, the viral types most closely associated with cervical carcinoma.

Materials and Methods

Cell Culture. From American Type Culture Collection (Rockville, MD) we obtained human cell lines CaSki, SiHa, and HeLa that contain HPV 16 or HPV 18 sequences (1). They were grown in DME supplemented with 10% FCS (Gibco Laboratories, Grand Island, NY).

Polymerase Chain Reaction (PCR). The PCR was run essentially as described (5) on a modified liquid handling apparatus (Pro/Pette; Cetus Corp., Emeryville, CA) which sequentially denatured, cooled, and added DNA polymerase I (Klenow; US Biochemicals, Cleveland, OH) to the samples. Oligomer primers, designated in Fig. 1, were synthesized on a DNA synthesizer (model 380; Applied Biosystems, Inc., Foster City, CA). The concentrations of the oligomers were 1.0 μM when a single pair were used and 0.75 μM when both pairs of oligomers were used. Each sample was amplified for 20–40 9-min cycles. The final reaction products were immobilized on a Gentrans 45 nylon filter (Plasco, Woburn, MA) and detected with a ^{32}P end-labeled oligomer probe (10^8 cpm/ μg , 2 ng/ml) in a dot blot assay. The filter was prehybridized for 1 h at 42°C in 3 \times SSPE (1 \times SSPE: 0.18 M NaCl, 10 mM NaH_2PO_4 , 1 mM EDTA, pH 7.4), 5 \times Denhart's, 0.5% NaDodSO₄ before adding the ^{32}P end-labeled oligomer and hybridizing for 1 h at 42°C. The filter was washed in succession with 1 \times SSPE with 0.1% NaDodSO₄, three times for 5 min at room temperature, once at 55°C for 10 min when washing the PCR with only H1 and H2 as primers, or once at 60°C for 10 min when washing the PCR with H1, H2, and H3 as primers, and then once for 5 min at room temperature. Autoradiography was with two intensifying screens (Cronex Hi Plus; DuPont Co., Wilmington, DE) at -70°C with Kodak X-AR film.

Paraffin Sections. Paraffin-embedded tissue blocks from patients at the Los Angeles County–University of Southern California Medical Center were classified based on standard morphologic criteria. They were up to 6 mo old and selected at random. The tissues, fixed in 10% buffered formalin, were processed by an Autotechnicon (model 2A Technicon Instruments Corp., Tarrytown, NY). A single 5–10- μm section cut from the block was placed in a 500- μl Eppendorph tube. Subsequent sections were stained with hematoxylin and eosin to confirm the exact tissue analyzed. The average surface area of the tissue was 0.4 cm^2 , though smaller fragments were used successfully. The sections were deparaffinized by adding 400 μl of xylene, and then centrifuged for 5 min (model 5412; Brinkmann Instruments Co., Westbury, NY). The xylene was decanted and its residue was removed with 400 μl of 95% ethanol, centrifuging and decanting again. The tissue pellets were grey-white in color and either fragmented or intact in a curled sheet. They were desiccated and 100 μl of the PCR mix was added directly to the tubes. The tubes were heated at 100°C for 10 min and then subjected to 40 cycles of amplification.

Results and Discussion

The HPV 16 and 18 genomes have been sequenced (10, 11). They contain several open reading frames. We chose to amplify a segment of the open reading frame designated as E6 (Fig. 1). The E6 gene is expressed in human cervical carcinoma cell lines (1, 12) and may code for a transforming protein. The E6

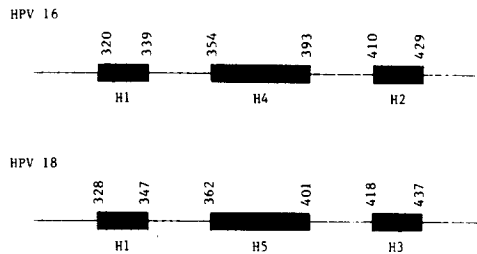


FIGURE 1. The oligomer primers and probes used in the HPV PCR are shown. They correspond to sequences homologous to the E6 open reading frame of both HPV 16 and 18. The base positions are numbered from their published sequences (10, 11).

H1 5' ATTAGTGACTATAGACATTA3'
 H2 5' GCGTTTTGCAGTTAATACA3'
 H3 5' GGTTCCTGGCACCAGCA3'
 H4 5' ATGGAACAACATTAGAACAGCAATACAACAACCCCTTG3'
 H5 5' ATCGGAGACATTGGAAAACTAACTAACACTGGGTTATA3'

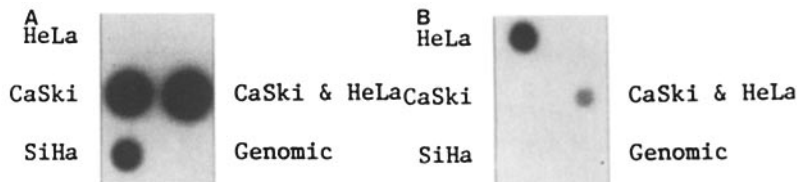


FIGURE 2. DNA (1.0 μ g) isolated from HeLa (10–50 copies HPV 18 per cell) or SiHa and CaSki (1–2 and 600 copies HPV 16 per cell) was subjected to 20 cycles of amplification with primers specific for HPV 16 and 18. The reaction products were hybridized against (A) H4 (HPV 16) or (B) H5 (HPV 18). Specific amplification was demonstrated and the reaction products from the sample containing both CaSki and HeLa DNA (0.5 μ g each) hybridized with H4 and H5. Equivalent amounts of pre-PCR DNA gave much weaker signals with their appropriate probes after the 1 h of autoradiography (not shown).

gene sequences of the papilloma viruses associated with cervical carcinoma (HPV 16 and HPV 18) are more similar to each other than they are to the papilloma viral types not associated with cervical carcinoma. The region amplified included the E6* intron, which is present in HPV 16 and 18 but absent in HPV 6 and 11 (12). The oligomers were designed to specifically detect HPV 16 or 18 as defined by their respective DNA sequences with specificity determined by both the primers used for amplification and the oligomers used for detection. A common 5' primer (H1) was used for both HPV 16 and 18. It was completely homologous to the HPV 16 sequence and 80% homologous to the HPV 18 sequence, with complete homology to the 8 bases of the terminal 3' portion of the sequence. The 3' primers for HPV 16 (H2) and 18 (H3) were different at 9 of 20 positions and correspond to their published sequences. The detecting oligomers, H4 for HPV 16 and H5 for HPV 18, corresponded to their published sequences and were only 60% homologous.

DNA was isolated from cell lines containing HPV 16 (CaSki and SiHa, ~600 and 1–2 copies of HPV 16 integrated in their respective genomes [1]) or HPV 18 (HeLa, 10–50 copies of HPV 18 integrated in its genome [1]). 1 μ g of DNA was subjected to the HPV PCR in the presence of primers for both HPV 16 (H1, H2) and HPV 18 (H1, H3). After amplification, the reaction mixtures were blotted on two separate filters and hybridized against labeled HPV 16-specific

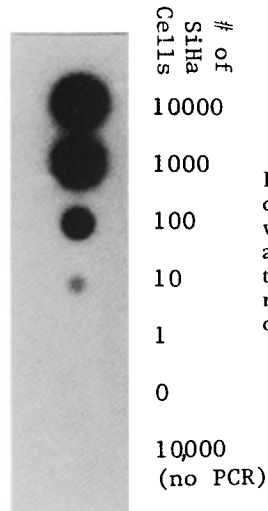


FIGURE 3. The sensitivity of the HPV PCR was demonstrated by serially diluting SiHa cells (1–2 copies HPV 16 per cell) in water. The samples were placed in a boiling water bath, and then subjected to 40 cycles of amplification with the primers specific for HPV 16. The figure indicates the number of cells added to each reaction tube. After 1 h of autoradiography, 10 SiHa cells corresponding to 10–20 copies of HPV 16 could be detected. 10,000 unamplified SiHa cells could not be detected.

probe H4 (Fig. 2A) or labeled HPV 18-specific probe H5 (Fig. 2B). These results were obtained after 1 h of autoradiography. Both the CaSki and SiHa reaction products hybridized to H4 but no hybridization with the reaction products from HeLa or human genomic DNA was detected. When hybridized against H5, only HeLa reaction products were detected. The reaction products from 0.5 μ g of CaSki DNA and 0.5 μ g of HeLa DNA hybridized to both H4 and H5. When the entire genomes of HPV 6b and 11a, inserted into pBR322, were similarly tested, faint hybridization could be detected only after 24 h of autoradiography (data not shown) and were clearly distinguishable from the much stronger homologous signals.

The sensitivity of the HPV PCR was determined with a serial dilution of SiHa cells. To avoid loss of DNA associated with phenol-chloroform purification procedures and to demonstrate the use of the HPV PCR on unpurified samples, the cells were boiled in water for 5 min and then subjected to the PCR with H1 and H2 as primers. 10 SiHa cells corresponding to 10–20 copies of HPV 16 could be detected (Fig. 3). No hybridization signal could be seen with 10^4 cells not subjected to amplification.

The CaSki cell line was fixed with formalin and paraffin embedded. A 7- μ m section containing $\sim 1,000$ cells ($\times 10^5$ copies of HPV 16) was deparaffinized and subjected to the PCR with H1 and H2 as primers. A strong signal was seen after 75 min of autoradiography. We next attempted to detect HPV 16 or 18 in tissue samples embedded in paraffin (Fig. 4). Paraffin-embedded tissues not expected to contain HPV (kidney, normal cervix) did not show evidence of amplification with the PCR. 10 tissue sections more likely to contain HPV were subjected to the HPV PCR. They consisted of nine cervical biopsies and one penile squamous cell carcinoma. The positive samples gave signals several-fold stronger than the amplified controls consisting of 100 HeLa or SiHa cells. All of the cervical biopsies were positive for either HPV 16 or 18. No dual infections were detected. Three of the biopsies showed severe dysplasia (CIN III), all of which were positive for HPV 16. The remaining six cervical biopsies showed invasive

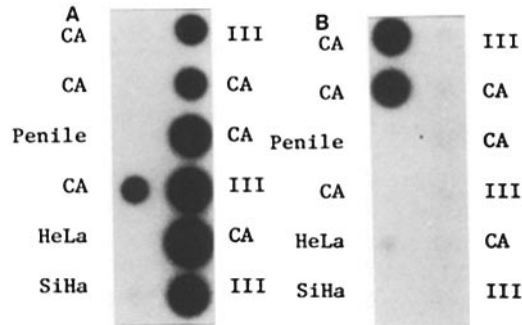


FIGURE 4. HPV PCR on paraffin-embedded tissue. The sections were subjected to 40 cycles of amplification with primers specific for HPV 16 and HPV 18. The reaction products were hybridized against (A) H4 (HPV 16) or (B) H5 (HPV 18). After 1 h of autoradiography, the controls consisting of SiHa or HeLa (100 amplified cells) were appropriately positive or negative with strong signals present from the tissue sections. (CA) cervical squamous cell carcinoma, (III) severe cervical dysplasia, (Penile) penile squamous cell carcinoma.

squamous cell carcinoma, four positive for HPV 16 and two positive for HPV 18. The single penile squamous cell carcinoma was negative for HPV 16 or HPV 18. The entire assay on paraffin embedded tissue could be conducted in <24 h.

The results reiterate previous studies using conventional DNA probe techniques linking HPV 16 and 18 infections with the majority of cervical carcinomas (1, 2). The technique is ostensibly *in situ* as the exact lesion under investigation can be correlated with a hematoxylin and eosin section taken from an adjacent 5 μ m slice. HPV 16 or 18 was detected from the entire tissue section, though microdissection of the tissue block could probably localize the amplification to epithelial cells. A positive HPV PCR signal does not necessarily indicate the presence of the entire HPV 16 or 18 genome, but does identify the presence of sequences thought important to their carcinogenesis.

A striking feature of the study is the relative ease with which paraffin-embedded tissue can be studied using the PCR. Initial experiments attempting to extract the DNA from paraffin blocks before amplification were time consuming and inconsistent. Excellent results were seen when the entire deparaffinized tissue slices were added to the reaction tubes. The cyclic heating of the reaction tubes during the PCR apparently allows segments of DNA to become available to the DNA polymerase. We have successfully applied the PCR to embedded tissues up to 40 yr old. The convenience and rapidity of this procedure should facilitate accrual of epidemiologic data relating HPV 16 and 18 infections with cancer.

The PCR technique can potentially detect the presence or absence of any defined DNA sequence. Paraffin-embedded tissues are a vast reservoir of both archival and daily specimens, representing the entire spectrum of human pathology. Hypotheses linking the presence or absence of specific DNA sequences, such as viruses, oncogenes, antioncogenes, or mutated genomes, to biologic phenomena can be rapidly tested with the appropriate primers and detection systems. The ability to detect the presence or absence of informative DNA sequences from a single slice of paraffin-embedded tissue represents a step towards linking histology, the basis of traditional surgical pathology, with advances in molecular biology.

Summary

Human papilloma virus (HPV) DNA sequences have been detected in paraffin-embedded tissue using an enzymatic *in vitro* amplification technique known as

the polymerase chain reaction. Amplification of a HPV DNA sequence before its detection with a cDNA probe significantly increases the rapidity as well as the sensitivity of detection such that a single 5–10- μ m thick paraffin-embedded tissue section can be analyzed within 24 h. The assay specifically detected HPV 16 or 18 without crossreactivity with HPV 6 or 11. As few as 20 viral copies could be detected. The rapid and sensitive analysis of HPV in normal and pathological tissues using this technique may contribute significantly to identifying the role of HPV as a risk factor in carcinoma.

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