Detection and Typing of Human Papillomavirus in Archival Cervical Cancer Specimens by DNA Amplification With Consensus Primers

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We developed a polymerase chain reaction DNA amplification system using two distinct consensus oligonucleotide primer sets for the improved detection and typing of a broad spectrum of human genital papillomavirus (HPV) sequences, including those of novel viruses. The system incorporates one primer set designed to amplify a highly conserved L1 domain and a second primer set designed to amplify a domain within the E6 gene. We used this system to analyze 48 fixed, paraffin-embedded tissue sections (41 specimens from 33 cervical carcinomas, four normal cervical tissues, and several control tissues) for the presence of HPV DNA. HPV sequences were detected in all carcinoma samples and none of the control samples. Hybridization analyses showed that the results obtained with the two amplification schemes concurred completely. This approach allowed rapid confirmation of typing results and may improve the likelihood of detecting a wide variety of HPV sequences, including those of novel HPVs. [J Natl Cancer Inst 82:1477–1484, 1990]

Over 60 distinct types of human papillomaviruses (HPVs) have been identified and can be categorized by their predilection for distinct mucosal or dermal sites. Genital (or mucosal) papillomaviruses are strongly associated with the development of benign condylomas and cervical carcinomas [for reviews, *see* (*1–3*)]. The presence of certain HPV types in premalignant lesions and in invasive cancers of the cervix implicates these HPVs in the etiology of neoplasia [for reviews, *see* (*4,5*)]. For instance, HPV types 16 and 18 and, to a lesser extent, HPV types 31, 33, 35, and 45 are found in invasive cervical carcinomas and in their metastases, whereas HPV types 6 and 11 are typically found in benign condylomas. Moreover, most, if not all, cervical squamous cell carcinomas contain HPV DNA sequences. Despite a strong correlation between the presence of certain HPV types and the development of anogenital cancers, the exact role of these viruses in the progression of these various clinical manifestations is not well understood. Sensitive methods for the detection and typing of HPVs in a large number of dysplastic tissues of different grades will be important tools for understanding the role of HPVs in tumorigenesis.

Many current methods for HPV detection are based on DNA hybridization (*6*). These methods vary widely in the time and expertise required to perform them and in their sensitivity. To assess the overall utility of these techniques in routine screening for HPV, several studies have directly compared the sensitivities and specificities of these techniques (*7–11*). In situ hybridization of DNA or RNA in tissue sections and cervical smears allows direct and exact visualization of infected cells in a microscopic field with a sensitivity of 50–200 copies per cell; however, this procedure is time consuming and labor intensive. Filter in situ hybridization techniques to detect HPVs in exfoliated cervical cells, as well as dot blot hybridization techniques, require little preparation of samples and are rapid, but they are incapable of detecting fewer than 10,000 copies of HPV DNA.

Southern blot hybridization, considered the gold standard in the field, provides comparable sensitivity to dot blot hybridization and provides valuable information about the genetic structure of the virus genome and its state within the host cell. However, it is considerably labor intensive and is subject to interlaboratory variability (*12*), thereby precluding its usefulness in routine screening. Furthermore, it requires significant amounts of tissue and provides little information about the histology of the sample. Finally, it is not particularly useful in the analysis of DNA from archival tissues, which is often fragmented or highly modified as a consequence of fixation (*13*).

Polymerase chain reaction (PCR) amplification allows rapid and specific detection of low-abundance viruses and single-copy genes in clinical specimens (*14–16*), even in cases in which the target DNA is of insufficient quality or quantity for other techniques (*17,18*). PCR amplification can be applied to sections of fixed, paraffin-embedded tissues and is a simple and highly sensitive technique for analyzing HPV DNA sequences in archi-

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val materials that can be correlated to the histology of adjacent sections (*7,8,19–22*).

Several amplification systems use separate, distinct consensus primer sets to amplify single HPV types that can be distinguished on the basis of amplification product size (*20,23–26*). Although such strategies are highly specific, they require different primer sets for each known viral type and are unlikely to detect sequences from novel or variant viral types.

We (*21,27*) and other researchers (*28,29*) have previously reported methods using consensus primers to amplify HPV DNA sequences. The consensus primers that we described are complementary to highly conserved sequences in the L1 region of genital HPVs. Using consensus and type-specific oligonucleotide probes in hybridization analysis of the amplification products, we were able to detect as few as 10 copies of HPV. In addition to amplifying many known genital HPVs (types 6, 11, 16, 18, 31, 33, 35, 39, 40, 42, 45, and 51–59), the L1 consensus primers amplify more than 25 other secondary or novel HPV types [(*21,27*); unpublished observations from the laboratory of Dr. Manos].

In this article, we introduce a second consensus primer set designed to amplify a 240-base pair (bp) region of the E6 gene. This approach provides a means to corroborate HPV typing data and focuses on a gene that is consistently retained and expressed in tumors (*30–32*). Archival tissue sections, which included specimens from 33 cervical carcinomas, were subjected to PCR amplification with the L1 and E6 primer sets. The resulting products were analyzed by dot blot hybridization with consensus and type-specific oligonucleotide probes to illustrate the efficacy of the two-site amplification strategy in the detection and typing of genital HPVs.

Materials and Methods

Control DNA

HPV DNAs used in control amplifications were either recombinant plasmids containing HPVs (types 6, 11, 31, 33, 39, and 45) or DNAs from cell lines containing HPVs. The cell lines were obtained from the American Type Culture Collection (ATCC), Rockville, Md. HPV16 was from the SiHa cell line (ATCC No. HTB 35) or from the CaSki cell line (ATCC No. CRL 1550), and HPV18 was from the HeLa cell line (ATCC No. CCL2). DNA from the K-562 cell line (ATCC No. CCL 243) served as an HPV-negative control. Cellular DNA (3–15 ng) or plasmid DNA (1–10 pg) was used in control amplifications. Products from these amplifications were used to assess the specificity of type-specific probes.

Southern Blot Analysis

DNA was extracted from 12 frozen tumor samples (specimens 1–12 in table 1). Purified DNA (5 μg) was digested with *Bam*HI restriction endonuclease and subjected to Southern blot analysis using an HPV16 or an HPV18 genomic probe under stringent conditions as previously described (*7*).

Tumor Samples

Sections from 48 paraffin-embedded tissue blocks representing 33 cervical carcinomas and seven HPV-negative control tissues were acquired from the pathology archives at the University of Amsterdam, the University of California at San Francisco, and the University of California at Davis. The cervical carcinoma specimens were obtained between 1979 and 1988. Sections (5 μm) were cut from tissue blocks with a microtome, and the microtome blade was thoroughly cleaned with xylene between each specimen to minimize cross-contamination of specimens. Dry sections were transferred to 1.5-mL microfuge tubes for PCR analysis, and immediately adjacent sections were mounted and stained with hematoxylin and eosin for independent histologic characterization.

Preparation of DNA From Paraffin-Embedded Tissues

The preparation of DNA from tissue sections for PCR amplification has been previously described (*33*). A single 5-μm section was deparaffinized by extraction with organic solvents and digested with 200 μL of a proteinase K solution. Procedures to prevent specimen contamination and PCR carryover were rigorously observed at every step in this analysis (*34,35*). They included interspersion of HPV-negative tissues with tumor specimens during tissue preparation for the detection of any possible contamination during specimen manipulation. The prepared specimens were used immediately for PCR amplification, although storage at −20 °C for up to 2 weeks resulted in only a nominal loss in the yield of the specific amplification product.

PCR Amplification

L1 and E6 amplification reactions were performed with 1 and 10 μL (0.5% and 5%, respectively) of each prepared specimen. In addition to HPV-negative and HPV-positive control samples, no-DNA controls (i.e., reaction mixtures to which no DNA was added) were included during each amplification series for the detection of contamination during reaction setup. Furthermore, to minimize contamination, we used only positive displacement pipettes and disposable pipettes in the assembly of amplification reactions.

Each L1 amplification reaction contained 50 pmol each of the L1 degenerate primers MY11 and MY09 (fig. 1), as previously described (*27*), with the following modification: 5 pmol each of the β-globin primers GH20 (5′GAAGAGCCAAGGACAGGTAC) and PC04 (5′CAACTTCATCCACGTTCACC) was included for the simultaneous amplification of a human β-globin product of 260 bp that served as an internal control.

The E6 reactions included 10 pmol of WD72 and 40 pmol of WD76 (the positive-strand primers) and 10 pmol of WD66, 40 pmol of WD67, and 10 pmol of WD154 (the negative-strand primers) (fig. 1) in a buffer containing 50 m*M* KCl, 10 m*M* Tris (pH 8.3), 4 m*M* $MgCl_2$, 200 μ*M* concentration of each deoxyribonucleotide triphosphate, and 2.5 U of AmpliTaq recombinant *Taq* polymerase (Perkin-Elmer Cetus Instruments, Norwalk, Conn) (*36*).

Each reaction was subjected to 40 amplification cycles in a DNA Thermal Cycler (Perkin-Elmer Cetus Instruments), using thermocycle-step parameters of 95 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 2 minutes. An additional 5 minutes was included at the final 72 °C elongation cycle. Thirty cycles were used in the amplification of HPV-positive control DNA. Amplification products (one twentieth of the reaction) were separated

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by electrophoresis using 7% polyacrylamide gels and were visualized by UV illumination following ethidium bromide staining.

Probes

Sequences of HPVs from the literature (*37–43*) and from our unpublished studies (laboratory of Dr. Manos) were used in the design of type-specific probes. Oligonucleotide probes used for the dot blot hybridizations were labeled to a specific activity of 0.3 to 1 × 10^7 cpm/pmol with [γ-^{32}P]adenosine triphosphate with T4 polynucleotide kinase (*44*).

The consensus L1 probe was an equimolar mixture of the following four oligonucleotides corresponding to a conserved region located 50 bp from the 5′ end of the amplified region: MY18 (5′CTGTTGTTGATACTACACGCAGTAC), MY46 (5′CTGTGGTAGATACCACWCGCAGTAC), MY57 (5′CTGTGGTAGATACCACACGTAGTAC), and WD147 (5′CTGTAGTGGACACTACCCGCAGTAC). W denotes A + T.

L1 type-specific probes (MY12 for HPV6, MY13 for HPV11, MY14 for HPV16, WD74 for HPV18, and MY16 for HPV33) were previously described (*27*). MY12 and MY13 were mixed together to detect both HPV6 and HPV11. L1 type-specific probes not previously described were used: Probes WD126 (5′CCAAAAGCCCAAGGAAGATC) and WD128 (5′TTGCAAACAGTGATACTACATT) allowed detection of HPV31, MY59 (5′AAAAACAGTACCTCCAAAGGA) was an additional probe specific for HPV33, and MY70 (5′TAGTGGACACTACCCGCAG) was specific for HPV45. Probes WD126, WD128, and WD170 were designed with sequence information generated from amplification products of clinical isolates that were subsequently identified as HPV31 and HPV45 by restriction enzyme digestion patterns (*21*). PC03 (5′ACACAACTGTGTTCACTAGC) was used to identify the β-globin product.

The following oligonucleotide probes were designed to distinguish E6 products: WD133 (5′ACACCTAAAGGTCCTGTTTC) at nucleotide 248 in HPV6, WD134 (5′ACACTCTGCAAATTCAGTGC) at nucleotide 175 in HPV11, WD103 (5′CAACAGTTACTGCGACG) at nucleotide 206 in HPV16, WD170 (5′GCAAGACATAGAAATAA) at nucleotide 178 in HPV18, WD165 (5′AAATCCTGCAGAAAGACCTC) at nucleotide 116 in HPV31, RR1 (5′GTACTGCACGACTATGT) at nucleotide 96 in HPV33, and WD171 (5′ACAAGACGTATCTATTG) in E6 of HPV45. The sequence of the HPV45 E6/E7 region was determined from the pHPV45 plasmid (provided by K. Shah, The Johns Hopkins University, Baltimore, Md). The following additional probes were designed, and their specificity was confirmed by hybridization: WD132 (5′GACAGTATTGGAACTTACAG) at nucleotide 213 in HPV18, WD166 (5′CCTACAGACGCCATGTTCA) at nucleotide 248 in HPV31, and RR2 (5′ACCTTTGCAACGATCTG) at nucleotide 212 in HPV33.

Dot Blot Hybridization of PCR Products

Replicate dot blots were prepared using one fiftieth (2 μL) of each amplification reaction. Additionally, control reactions (HPV-positive SiHa DNA, HPV-negative K562 DNA, and no DNA) from each amplification series were included. Two microliters of each reaction was denatured in 100 μL of 0.4 *N* NaOH and 25 m*M* EDTA (edetic acid) for each dot, applied to a Gentrans 45 membrane (Plasco, Inc., Woburn, Mass), and covalently bound by UV linking using a Stratalinker (Stratagene, Inc., San Diego, Calif) at 400 (×100) μJ. The filters were washed in 0.1× standard saline citrate[1] (SSC) and 0.5% sodium dodecyl sulfate (SDS) at 60 °C for 30 minutes, followed by prehybridization for 15 minutes at 55 °C with 6× SSC, 5× Denhardt's solution,[2] 0.5% SDS, and 100 μg of single-stranded, sheared salmon sperm DNA per milliliter.

Replicate filters were separately hybridized with ^{32}P-labeled, type-specific and generic oligonucleotide probes (100,000–200,000 cpm/mL) in 6× SSC, 5× Denhardt's solution, 0.5% SDS, and 100 μg of single-stranded, sheared salmon sperm DNA per milliliter for 1 hour at 55 °C. Probes WD170 and WD171 required hybridization at 45 °C.

Filters were rinsed briefly in 2× SSC and 0.1% SDS at room temperature and then twice for 10 minutes at 45 °C (WD170 and WD171), 50–52 °C (WD132, RR1, and RR2), 55–56 °C (the consensus probe mixture, WD103, WD132, WD165, and WD166), 56–57 °C (MY12/13, WD126, MY16, MY70, and WD133/134), or 58–59 °C (MY14 and WD74). The membranes were subjected to autoradiography using Kodak XAR-5 film. Results from L1 and E6 dot blots were scored independently.

Results

Design of Amplification Primers and Probes

We have previously shown the efficacy of a PCR amplification strategy using consensus primers to amplify part of the L1 region from a variety of genital HPV types (*21,22,27*). Although amplification from fresh clinical specimens or paraffin-embedded tissues posed no overt problems, we were concerned about the prospect of false negatives arising from specimens bearing aberrant HPV L1 sequences. Therefore, we sought to improve the basic methodology.

We designed a second primer set to amplify a smaller fragment that was in a different region of the HPV genome. We reasoned that this second primer set would (*a*) improve our basic system by providing confirmational typing data for each sample, (*b*) favor amplification of an HPV sequence from samples containing highly degraded DNA, and (*c*) afford detection of HPV sequences in the event L1 sequences are altered or disrupted. We chose the E6 gene as the site for amplification, because this gene, along with E7, is retained and expressed in malignant tumors.

Although, based on heteroduplex mapping, the E6/E7 region is not as highly conserved in sequence as the L1 gene domain (*45*), two conserved regions of about 20 bp each were identified by sequence alignment (fig. 1). We then designed consensus primers at these sites to amplify a 240- to 250-bp fragment by using a variety of HPV templates. While the final selection of efficient and specific primers was empirically determined from several possible variations, the approach used in their design followed some basic tenets. Primers were designed to be 17–20 nucleotides in length with an average G + C content near 50%, and sequences with complementarity or secondary structure potential were

[1] 1× SSC = 0.15 *M* sodium chloride and 0.015 *M* sodium citrate (pH 7.0).

[2] 50× Denhardt's solution = 1% bovine serum albumin, 1% Ficoll 400, and 1% polyvinylpyrrolidine.

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avoided. The positive-strand primers (WD72/76) reside just upstream of the open reading frame of the E6 gene and contain part of a repeated sequence element ($ACCN_6GGT$) found in the upstream regulatory regions of many papillomaviruses and thought to be a target for the E2 protein [for review, *see* (*46*)]. The negative-strand primers (WD66/67/154) are located in the E6-coding region. The primers WD76 and WD67 contain two and five degenerate positions, respectively, to accommodate a variety of HPV templates. However, to ensure the amplification of HPV18, we included specific primers WD72 and WD66. Furthermore, we added WD154 because the sequence of HPV31 differed significantly from that of the other viruses in the region of the negative-strand primers (fig. 1). Amplification of both HPV31 and HPV39 required the inclusion of this primer. The amplified fragment contains sequences encoding the first of four regularly spaced (C–X–X–C) cysteine doublets, which are homologous to the zinc-binding motifs and implicated in the function of transcriptional activators (*47*). The amplified region also contains an alternative splice donor site, which is responsible for the spliced E6* transcript identified from HPV types frequently associated with malignancies (*48*).

Following optimization of the primer and $MgCl_2$ concentrations and the cycling parameters, the primer set was tested for its ability to produce an E6 amplification product from several genital HPV types. The optimal primer ratios for efficient amplification of a broad spectrum of HPV templates were determined empirically. Figure 2A shows that the amplification of a variety of HPV DNAs produced fragments of about the expected size. Differences in the predicted product sizes, calculated from published HPV sequences, accounted only in part for the mobility differences observed in the polyacrylamide gels. (We often observed aberrant mobilities in acrylamide gels.) The doublets observed in some E6 amplifications were likely to result from secondary priming at the repeated $ACCN_6GGT$ motif. The primers amplified HPV types 6, 11, 16, 18, 31, 33, 39, and 45, in addition to HPV types 42 and 52 (data not shown).

We used the E6 primers to amplify HPV sequences in a collection of paraffin-embedded cervical cancer specimens (table 1). The right panel of figure 2A shows examples of E6 amplification products from five specimens of squamous cell carcinoma (including two lymph node metastases), two specimens of adenocarcinoma, normal cervical tissues, a negative tissue (appendix), and a positive control (≈200 copies of HPV16). Six specimens (27a, 13a, 21, 33, 14b, and 28) clearly yielded products of the expected size (225–246 bp). Products were also faintly visible in specimens 14a and 32. Both specimens 14b and 14a (sections from the primary tumor and the metastatic internal iliac lymph node of the same patient, respectively) produced E6 amplification products. Products of the expected size were not readily apparent in reactions from the normal cervix (specimens 34 and 35) or from appendix tissues (specimen 38).

Clinical specimens and controls were also amplified with the HPV L1 consensus primers, and the results were compared with those obtained with the E6 amplification. To assess DNA integrity and the relative quantity of DNA in the sample, we simultaneously amplified a region of the human β-globin gene. The amplified β-globin fragment (268 bp) was visible in ethidium bromide-stained gels (fig. 2B). HPV amplification products of the expected size were observed in most tumor specimens. Again, both the primary tumor and the metastasis of specimen 14 produced HPV products. In five tumor specimens, the DNA integrity or yield was compromised, and these specimens failed to yield β-globin gene amplification products. Most of the prepared tumor specimens (41 of 46) produced the β-globin fragment, and

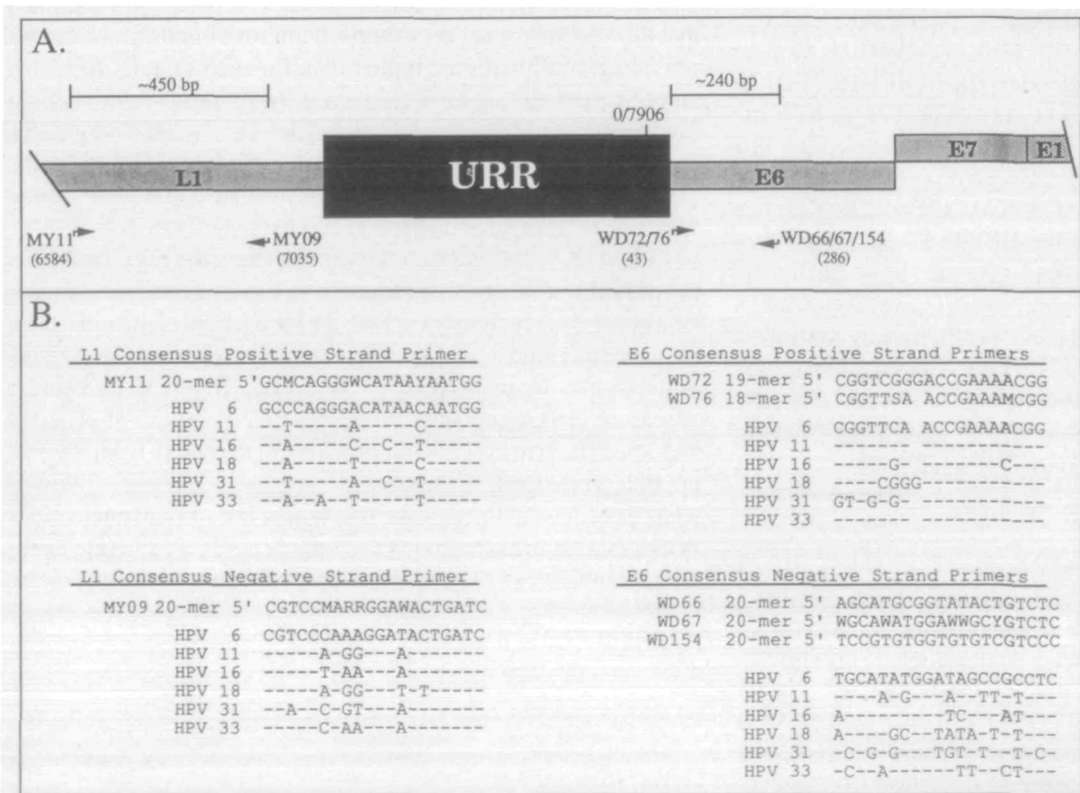

Figure 1. Consensus primer sets. (A) Locations of consensus primers are shown below the partial map of HPV16. Open reading frames and the upstream regulatory region (URR) are depicted by shaded boxes. Numbering on map corresponds to HPV16, and position of 5′ end of each primer in the HPV16 sequence is shown in parentheses below each primer. (B) Alignment of six genital HPV sequences (*37–43*) corresponding to consensus primers. Primer sequences are shown with HPV sequences aligned below. Dashes represent nucleotides that match the HPV6 sequence, and mismatches are shown at their respective positions. W = A + T; S = G + C; R = A + G; Y = C + T; M = A + C.

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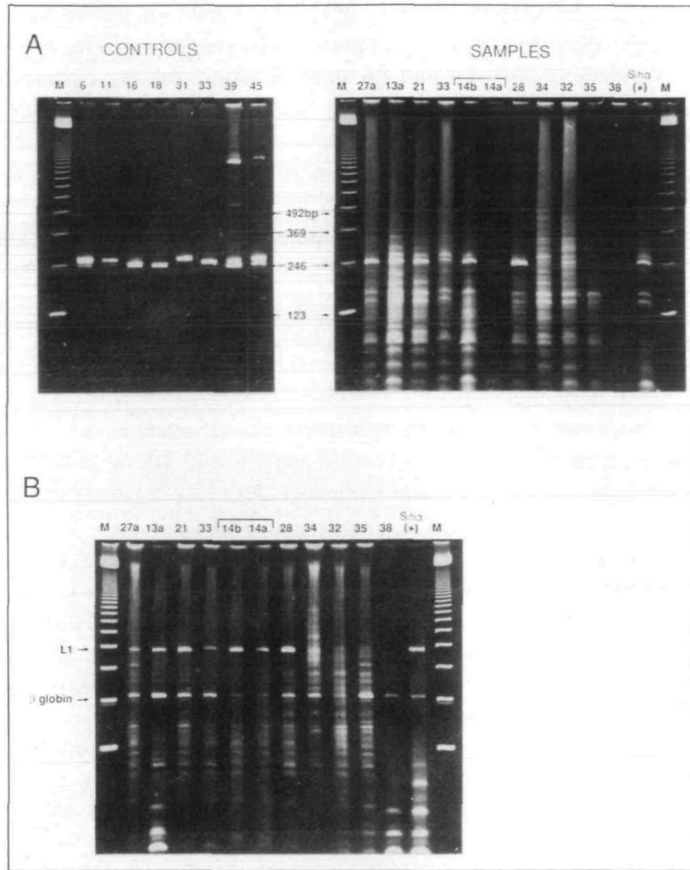

Figure 2. Gel electrophoresis of PCR products. Each reaction (one-twentieth) was visualized by ethidium bromide staining and photography under UV light following electrophoresis in a 7% polyacrylamide gel. DNA markers (123-bp ladder; GIBCO, Grand Island, NY, and Bethesda Research Laboratories, Inc., Gaithersburg, Md) are shown in lanes marked M. (A) E6 amplification products are shown. Lanes marked 6, 11, 31, 33, 39, and 45 are from HPV-containing plasmids, 16 is from CaSki cell DNA, and 18 is from HeLa cell DNA. Panel at right shows E6 PCR products from clinical specimens. Numbers above lanes correspond to specimen numbers described in table 1. (B) L1/β-globin PCR products from clinical specimens described in panel A. Positions of L1 (450 bp) and β-globin (268 bp) products are shown by arrows.

only these specimens were considered appropriate for study and were included in the results summarized in table 1. All tumor specimens determined to be sufficient for analysis (visible β-globin amplification) were positive for HPV.

In several specimens (e.g., specimen 32 from an adenocarcinoma and specimens 34 and 35 from normal cervices in figs. 2A and 2B), the presence of HPV amplification products was difficult to assess from the gels because of a high background of other reaction products. In such cases, we had to perform Southern blot hybridization to visualize amplification products of the expected size (not shown). Nonspecific background amplification bands were frequently observed in amplifications from the samples of paraffin-embedded tissues. These bands were most probably a consequence of target DNA integrity and nonspecific priming, which becomes increasingly detectable with more than 35 amplification cycles, since such backgrounds were not observed in control amplifications. In the final compilation of the data with every specimen, positivity was ultimately determined by the dot blot hybridization.

Table 1. Detection and typing of HPV in archival cervical tumor specimens*

Diagnosis	Specimen No.†	PCR results‡		Southern blot results§	
		L1	E6	HPV16	HPV18
Squamous cell carcinoma	1 2 3 4 5 6 7	16	16	+	−
	8	16	16	−	−
	9	18	18	−	+
	10	31	31	−	−
	11	33	33	−	−
	12	—	18	−	−
	13a‖ b 14a‖ b c d 15a b 16a b 17 18 19 20 21 22 23 24 25 26	16	16		
	27a b 28 29	18	18		
Adenocarcinoma	30a b 31 32	16	16		
	33	45	45		
Control tissues					
Cervix	34 35 36 37	—	—		
Appendix	38				
Kidney	39				
Spleen	40				

*Only specimens producing a visible β-globin amplification product were included in the final compilation of the data.

†Sections are from primary tumor except where designated. Sections from different spatial aspects of primary tumor were available in specimens 14-16, 27, and 30.

‡Nos. in columns are HPV type Nos. — = negative for HPV by L1 and E6.

§+ = positive for HPV type; − = negative for HPV type.

‖Sections from internal iliac lymph node metastasis.

Dot Blot Hybridization

The E6 and L1 amplification products were independently analyzed by dot blot hybridization with HPV-specific probes. The results obtained with the two schemes were then directly compared.

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The left panel of figure 3A illustrates the specificity of the E6 probes with control amplifications. Each probe detected only the type for which it was designed. Column K, representing amplification products from HPV-negative cellular DNA, demonstrated no detectable background hybridization with our HPV-specific probes. The right panel of figure 3A shows the hybridization analysis of E6 amplification products corresponding to the samples of paraffin-embedded tissues shown in figure 2. Both the primary tumor and the metastasis of specimen 14 contained HPV sequences identified as type 16. Additionally, specific HPV sequences were easily detected in specimens 32 and 33, which had appreciable background products in gel electrophoresis analysis. All the specimens defined as invasive cervical tumors by histopathologic examination in adjacent sections were found to contain HPV sequences. Moreover, we found only one HPV type per tumor. None of the specimens from the collection were found to contain HPV6 or HPV11. In repeated experiments, none of the control specimens produced HPV amplification products.

Figure 3B shows the hybridization analysis of the L1/β-globin amplification reactions. The left panel illustrates the specificity of the probes. The type-specific probes were highly discriminating and did not display cross-hybridization to PCR products of other types. The right panel shows the hybridization results from the tissue specimens described in figure 2. The β-globin product (Globin) was detectable by hybridization in all specimens. Only the cervical tumor specimens contained HPVs, as evidenced by hybridization with the generic (consensus) and type-specific L1 probes. Again, we found only one HPV type per specimen. Amplification reactions from each of the HPV-negative control tissues and no-DNA controls were carried through all hybridization analyses and were negative for HPV sequences in repeated experiments.

Table 1 summarizes the findings from this study of archival paraffin-embedded cervical tumor specimens. Of the 41 specimens examined from 33 tumors, 40 yielded visible and/or hybridizable HPV L1 and E6 amplification products. Specimen 12 produced an E6 product, but failed to yield an L1 product. None of the normal cervical tissues or the other control specimens contained HPV sequences. The normal tissues did produce the β-globin product, indicating that the target DNA was intact and was of sufficient quantity for amplification. When multiple sections from different parts of the tumor were available (specimens 14–16, 27, and 30), the same HPV type was found. Similarly, in two specimens of squamous cell carcinoma, HPV of the same type was identified in both the primary tumor and the lymph node metastases.

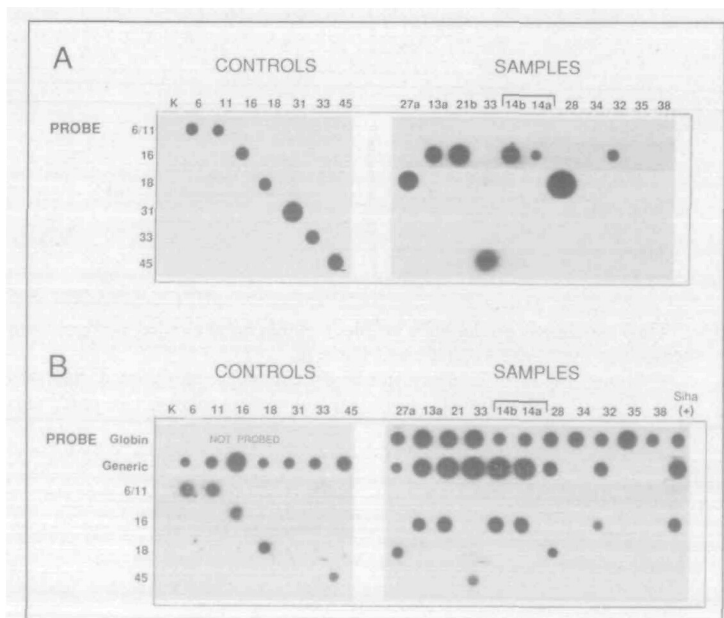

Figure 3. Dot blot hybridization analysis of PCR products. Of each amplification reaction, one fiftieth was applied to replicate filters and hybridized with ^{32}P-labeled oligonucleotide probes. Probes used for hybridization are shown to left of strips. (A) Analysis of E6 products. Each amplification sample was described in fig. 2. (B) Analysis of L1/β-globin PCR products. "Generic" designates L1 consensus probe described in Materials and Methods section.

With the exception of specimen 12, all specimens yielded confirmatory typing results with the E6 and L1 amplification schemes. Multiple sections from specimen 12 were analyzed and failed to produce either a visible or hybridizable HPV L1 PCR product. Other primer sets within the globin gene were used to test for the ability of this specimen to amplify fragments of a size similar to that of the L1 amplicon. Globin amplification products of 430 and 599 bp were easily produced (data not shown), suggesting that if the intact L1 target existed in the specimen, it could have been detected.

In specimens 1–12, DNA from a corresponding frozen tumor specimen was analyzed by stringent Southern blot hybridization using an HPV16 or an HPV18 genomic probe. PCR analysis revealed the presence of HPV in all 12 specimens, thereby confirming all positive findings determined by Southern blot analysis. The additional HPV-positive specimens found by PCR included two apparently missed by Southern blot analysis (specimens 8 and 12), illustrating the increased analytic sensitivity of the amplification methods (table 1).

Table 2 summarizes the distribution of HPV types found in this collection of tumors. In the 29 squamous cell carcinomas and four adenocarcinomas examined in this study, a variety of HPV types were found. HPV16 was the most prevalent in both types of tumors in this collection (22 of 29 squamous cell carcinomas and three of four adenocarcinomas). HPV18 was found in five squamous cell carcinomas, and single cases of HPV31, HPV33, and HPV45 were found.

Discussion

We have described a second consensus HPV primer set and type-specific probes to complement a consensus primer amplification in the HPV L1 region. We illustrated the use of this combined strategy in the study of HPV prevalence in archival cervical tumor specimens obtained 1–10 years before the study.

Table 2. HPV types in archival cervical tumors

Type of tumor	HPV types*					
	6/11	16	18	31	33	45
Squamous cell carcinoma (n = 29)	0	22	5	1	1	0
Adenocarcinoma (n = 4)	0	3	0	0	0	1

*Values = No. of tumors.

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The E6 gene was chosen as the site for the second consensus primer set, since the E6 and E7 genes are generally intact and expressed in cervical tumors. The intact open reading frames of these two genes are required for transforming activity in vitro and may be involved in tumor progression in vivo (*49*). Other regions were thought to be less attractive targets, since their sequences may be disrupted as a consequence of HPV integration (*37,50*). The amplification of the E6 region may allow the detection of such integrated viruses. Even in this small collection of tumors, one specimen failed to yield an L1 amplification product, whereas the E6 amplification was successful.

We also designed the second scheme to produce an amplification product smaller than that from the L1 system, in an effort to increase the likelihood of detecting HPV sequences in archival tissues. Although PCR methods have been used to amplify sequences from ancient DNA (varying in age from 4 to 13,000 yr), such DNA is sufficiently fragmented and modified to render it a poor template for amplification of products much longer than 140 bp (*18*). Amplification from paraffin-embedded tissues is subject to similar limitations. In separate studies (*51*), we have determined that the size of a fragment that can be efficiently amplified from fixed tissues is limited as a consequence of fixation time and fixative type. The inclusion of the scheme using the E6 gene, which yields a smaller amplification product than the L1 scheme, may help to circumvent this problem.

Finally, the use of two sites allowed rapid confirmation of HPV detection and typing results. The use of a second site may also circumvent the prospect of obtaining false-negative results as a consequence of sequence variation in one of the primer or probe-binding sites.

In a direct comparison of Southern blot analysis, we found that this system afforded greater sensitivity and required much less sample preparation. Amplification methods allowed significantly less sample DNA to be used compared with the 5–10 μg required for Southern blot analysis; visible β-globin and HPV amplification products were produced from the DNA present in 5-μm tissue sections. The results obtained with PCR confirmed those found by Southern blot analysis in most cases. The HPV16 detected in specimen 8 by amplification and the HPV18 detected in specimen 12 by amplification were missed by Southern blot analysis. Presumably, amplification allowed the detection of HPV in these specimens where the target DNA was of insufficient quality or quantity for detection by Southern blot analysis.

Typing results with the E6 and L1 schemes were in complete agreement in all specimens examined, except for specimen 12, which failed to produce an L1 amplification product. Although L1-negative, HPV-positive specimens have not been frequently observed by us or other researchers (Wheeler C: personal communication), the L1-negative specimen found in this study does illustrate the importance of a strategy based on a two-site analysis.

Although the number of specimens in this study was small, we found HPV DNA in each of the tumors, and the distribution of types found in the squamous cell carcinomas was consistent with that observed in other surveys. Although several studies have reported a high prevalence of HPV18 in adenocarcinoma (*52,53*), HPV18 was not detected in the small number of adenocarcinoma specimens that we analyzed. However, one of the adenocarcinoma specimens contained HPV45, the virus most closely related to HPV18 (*54*). As DNA amplification methods are used to study larger numbers of samples, it will be interesting to see if HPV DNA is present in all cervical carcinomas.

Although the E6 consensus primers directed the amplification of HPV types 6, 11, 16, 18, 31, 33, 39, 42, 45, and 52 as well as several unidentified HPV types, we have yet to determine whether they will amplify as broad a spectrum of viruses as the L1 consensus primers. Furthermore, we are currently improving the HPV-typing methods. For example, we are including a second oligonucleotide probe for each HPV type to circumvent the possibility of obtaining false negatives due to variants bearing bp deletions or substitutions within the probe sites. We have also recently developed a new generic probe composed of L1 products from four distinct HPV sequences. This probe affords the detection of a broader spectrum of HPVs than the generic oligonucleotide probe mixture used in the present study.

The ability to apply DNA amplification methods to studies of fixed, paraffin-embedded tissues provides molecular biologists and epidemiologists with a powerful tool to use vast collections of archival tissues of many tumor types. The consensus primer strategy presented here is one of the most efficient and sensitive methods for the detection of genital HPVs and for the characterization of novel virus types. This DNA amplification method may be a powerful approach for many retrospective studies concerning the role of HPV and other host genetic alterations leading to malignancy, changes in HPV prevalences over time, and the clinical utility of HPV typing.

Note added in proof: For information regarding DNA amplification from paraffin-embedded tissues, *see* references (*34*) and (*51*). Preprints of reference (*51*) are available from Dr. Manos.

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