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# Prevalence of Human Papillomavirus Types 16 and 18 in Cervical Carcinomas: A Study by Dot and Southern Blot Hybridization and the Polymerase Chain Reaction

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**Histologically classified biopsies from 83 women with invasive cervical carcinoma were analyzed by dot blot hybridization for human papillomavirus (HPV) types 16 and 18 infection. Sixty of the 83 (72.3%) were found to contain HPV DNA, of which 43 (51.8%) contained HPV 16 DNA, 12 (14.5%) contained HPV 18 DNA and 5 (6.0%) contained both HPV 16 and 18 DNAs. Southern blot analysis on 65 specimens gave similar results. Of 23 specimens negative by dot blot, 21 were tested by the polymerase chain reaction. Seventeen of the 21 were positive for HPV DNA, of which 13 contained HPV 16 DNA and 4 contained both HPV 16 and 18 DNAs. In all, 95.1% (77/81) were positive for HPV 16 and/or 18 DNA sequences.**

Key words: Human papillomavirus (HPV) 16 and 18 — Cervical cancer — Dot blot — Southern blot — Polymerase chain reaction

Epidemiological and molecular hybridization studies have demonstrated a close association of human papillomavirus (HPV) types 16 and 18 with invasive cervical carcinoma.[1-3] Wide geographical variation in the occurrence of these two HPV types in invasive cervical carcinomas has been reported. HPV 16 DNA was detected in 65% of invasive cervical carcinomas in Western Australia,[4] 61% in Germany,[5] 60% in Panama,[6] 57% in Austria,[7] 45% in Taiwan, China,[8] 40% in Italy,[9] 38% in England,[10] 35% in Kenya and Brazil,[5] 31% in Mexico,[11] 27–34% in Japan[12,13] and 18% in the United States.[14] The prevalence of HPV type 18 was much lower than that of HPV 16, being 25% in Kenya and Brazil,[5] 18% in Taiwan, China,[8] 15% Germany,[15] 9% in Austria,[7] 7% in Western Australia[4] and 6% in Japan.[12,13]

In most of these studies, the Southern blot hybridization procedure was used for the typing of HPV infection. Recently, the polymerase chain reaction (PCR) amplification method has been used to detect HPV infection in these patients. With this highly sensitive technique, the frequency of HPV infection in cervical cancer was found to range from 90–100%.[16-18]

We have determined the frequency of occurrence of HPV 16 and 18 DNA sequences in 83 histologically classified invasive cervical carcinomas using three hybridization techniques. The biopsied tissues were initially screened for the presence of HPV DNA by dot blot hybridization and subsequently confirmed by Southern blot. Specimens found to be negative for HPV by both methods were further analyzed using the PCR technique.

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## MATERIALS AND METHODS

**Specimen collection and DNA extraction** Colposcopically directed punch biopsy specimens were obtained from 83 patients with invasive cervical carcinoma seen at the Singapore General Hospital over a period of 9 months in 1987. The ages of the patients ranged from 33 to 83 years. One-half of the tissue sample was immediately snap-frozen in liquid nitrogen and stored until processed for DNA hybridization. DNA from biopsied tissue was extracted by the method of Davis *et al.*[19] The other half was formalinized for histological study. The histological classification of invasive cervical carcinoma followed the WHO criteria.[20]

**HPV DNA probe preparation** Recombinant plasmids containing full-length HPV DNA sequences of types 16 and 18 were kindly provided by Dr. H. zur Hausen (Heidelberg, Germany) and Dr. K. Hashimoto (Tokyo, Japan). The plasmids were introduced into *Escherichia coli* strain HB101 by transformation. DNA was purified from the vector with the appropriate restriction endonuclease (Bethesda Research Laboratories), followed by agarose gel electrophoresis and electroelution.[21]

The HPV DNA was labeled with $^{32}$P-labeled dCTP to a specific activity of $>1\times10^9$ cpm/$\mu$g DNA by using the multiprime DNA labeling system (Amersham) following the procedure of Feinberg and Vogelstein.[22]

**Dot blot hybridization** Cellular DNA was denatured in 0.25 *N* NaOH for 10 min, chilled on ice and diluted with 0.125 *N* NaOH and 0.125×SSC (1×SSC; 0.15 *M* NaCl and 0.015 *M* Na-citrate). The denatured DNA was transferred onto Gene Screen Plus™ nylon membranes

(DuPont) with the aid of a dot blot apparatus (Biorad). The membrane was pre-hybridized for at least 2 h in a solution containing 50% deionized formamide, 1% SDS, 1 *M* NaCl and 10% dextran sulfate at 42°C. The $^{32}$P-labeled HPV probe ($1\times10^6$ cpm/ml) was mixed with 200 $\mu$g/ml of denatured salmon sperm DNA (Sigma) and hybridization was carried out for 16 h at 42°C. The membranes were washed under stringent conditions, twice in 2×SSC at room temperature for 5 min, twice in 2×SSC and 1% SDS at 65°C for 30 min and then twice in 0.1×SSC and 0.1% SDS at room temperature for 30 min. Following hybridization, the membranes were exposed to Hyper film™-MP (Amersham) with Cronex Lightning Plus (DuPont) intensifying screens for 24–72 h at −70°C. For re-hybridization, the probes were removed from the membrane by boiling in 0.1×SSC and 1% SDS for 20–30 min. The membrane was re-exposed to X-ray film for up to 7 days to confirm that the radioactive probe had been removed. The copy number of HPV in biopsied specimens were estimated by comparing their hybridization intensity to that of CaSki and SiHa cell DNAs which were estimated to contain more than 500 and 1–10 copies of HPV 16, respectively, and HeLa cell DNA which has 50 copies of HPV 18 per cell.[23, 24]

**Southern blot hybridization** The DNA was cleaved with the appropriate restriction enzyme (2 units/$\mu$g DNA) and incubated at 37°C overnight. The DNA was electrophoresed on a 0.8% agarose gel and transferred onto a nylon membrane.[25] Hybridization was performed as described above.

**Oligonucleotide primers and probes for PCR** The segment of HPV genome chosen for amplification was the E6 open reading frame.[17] The primers and detecting oligomers were synthesized with an Applied Biosystems DNA synthesizer, model 381, and purified by high-performance liquid chromatography (Hitachi).

**PCR amplification of DNA** HPV sequences in biopsied tissue DNA were detected with separate pairs of primers, H1 (5′ATTAGTGAGTATAGACATTA3′) and H2 (5′GGCTTTTGACAGTTAATACA3′) for HPV 16 DNA and H1 and H3 (5′GGTTTCTGGCACCGCAGGCA3′) for HPV 18.

Genomic DNA (1 $\mu$g) was denatured by heating at 95°C for 5 min in 100 $\mu$l of reaction buffer containing 50 m*M* KCl, 10 m*M* Tris-HCl (pH 8.3), 1.5 m*M* $MgCl_2$, 200 m*M* dNTPs, 20 pmol of each primer and 2.5 units of *Thermus aquaticus* (Taq) polymerase (Perkin-Elmer Cetus).[26] Subsequent cycles consisted of primer annealing at 50°C (1 min 30 s) and primer extension at 70°C (2 min 15 s) carried out on a fully automated DNA thermal cycler (Perkin-Elmer Cetus Instruments). This cycle was repeated 29 more times, except that subsequent denaturations were done for 1 min 15 s instead of 5 min. After the last cycle, all samples were incubated for an additional 10 min at 70°C, to ensure completion of the final extension step.

The extension products were analyzed by electrophoresis on a 3% NuSieve (FMC) and 1% Agarose Type II: Medium EEO (Sigma) gel and stained with ethidium bromide. After photography, the DNA was transferred onto a nylon membrane. This was prehybridized as previously described. Hybridization was carried out with [$\alpha$-$^{32}$P]ddATP end-labeled (Amersham) oligomer probes H4 (5′ATGGAACAACATTAGAACAGCAATACAACAAACCGTTGTG 3′) for HPV 16 and H5 (5′ATGGAGACACATTGGAAAAACTAACTAACACTGGGTTATA 3′) for HPV 18 ($>2\times10^8$ cpm/$\mu$g, 2 ng/ml) for 16 h at 42°C.[18] The filter was washed in succession with 2×SSC and 0.1% SDS twice for 15 min and once in 2×SSC and 1% SDS for 30 min at 65°C. Autoradiography was done with intensifying screens at −70°C. The efficiency of the PCR reaction was calculated according to the method of Saiki *et al.*[27]

Due to the extreme sensitivity of the method, controls were included as a precaution against possible errors from extraneous DNA contamination. These controls were included in every experiment and subjected to exactly the same experimental conditions as the test specimens. The control positives used included HeLa and CaSki or SiHa cell DNA. Control negatives used were 2 Raji and P3HR-1 cell lines containing Epstein-Barr virus. A water control was also included to rule out possible contamination during amplification.

## RESULTS

**Dot blot hybridization** The sensitivity of dot blot hybridization was established by analyzing serial dilutions of a defined amount of purified HPV DNA. The limit of detection was found to be about 1.5 pg of HPV DNA.

Of 83 biopsied specimens, 60 contained HPV DNA detectable by dot blot hybridization (Table I). Forty-three of the 83 (51.8%) were positive for HPV 16 DNA, 12 (14.5%) HPV 18 DNA and 5 (6.0%) for both HPV 16 and 18 DNAs. Forty-two of 75 (56.0%) squamous cell carcinomas (SCC) contained HPV 16 DNA, 7 (9.3%) contained HPV 18 DNA and 5 (6.7%) contained both HPV 16 and 18 DNAs. Five of 7 (71.4%) adenocarcinomas were found to contain HPV 18 DNA, while the other 2 were negative. The specimens, which showed different degrees of intensity of hybridization with the HPV probes, were estimated to contain 1–160 copies of HPV DNA per cell by comparison with the DNA from cell lines with known copy numbers of HPV.

**Southern blot hybridization** Sixty-five biopsied specimens wtih adequate amounts of DNA were examined by Southern blot hybridization for the presence of HPV 16

Table I. Comparison of Dot and Southern Hybridization and the PCR for the Detection of HPV Types 16 and 18 DNA in Invasive Cervical Carcinomas

| Cervical carcinomas and tests | No. of specimens positive for HPV | | | |
|---|---|---|---|---|
| | HPV 16 alone | HPV 18 alone | HPV 16 and 18 | Negative |
| Adenocarcinoma | | | | |
| Dot blot | 0/7[b] | 5/7 (71.4) | 0/7 | 2/7 (28.6) |
| Southern blot | 0/6 | 4/6 (66.7) | 0/6 | 2/6 (33.3) |
| PCR[a] | 1/2 (50.0) | 0/2 | 0/2 | 1/2 (50.0) |
| Squamous cell carcinoma | | | | |
| Dot blot | 42/75 (56.0) | 7/75 (9.3) | 5/75 (6.7) | 21/75 (28.0) |
| Southern blot | 33/58 (56.9) | 7/58 (12.1) | 5/58 (8.6) | 13/58 (22.4) |
| PCR | 12/19 (63.2) | 0/19 | 4/19 (21.1) | 3/19 (15.8) |
| Undifferentiated carcinomas | | | | |
| Dot blot | 1/1 | 0/1 | 0/1 | 0/1 |
| Southern blot | 1/1 | 0/1 | 0/1 | 0/1 |

*a*) Polymerase chain reaction
*b*) No. positive/No. tested (%)

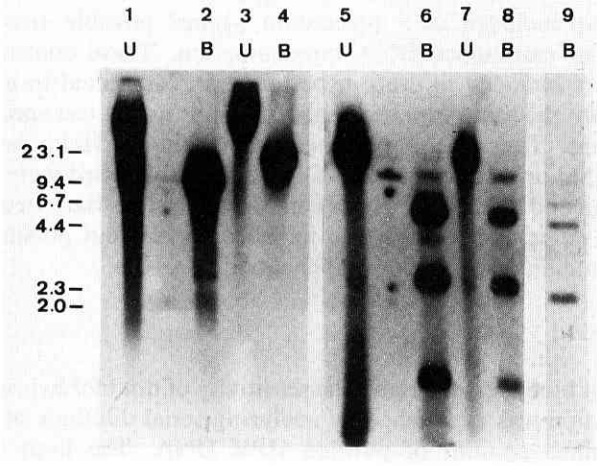


Fig. 1. Autoradiogram of Southern blots from 2 cervical carcinomas containing both HPV 16 and 18 DNA (lanes 1, 2, 5, 6 and 3, 4, 7, 8). DNA (10 $\mu$g) was digested with *Bam*HI (B) or not treated (U), and hybridized with HPV 16 (lanes 1–4) and HPV 18 (lanes 5–8) radiolabeled probes. A restriction profile of *Bam*HI-digested HeLa cells is shown in lane 9. Bars on the left represent size markers in kilobases of $\lambda$ DNA digested with *Hin*dIII.

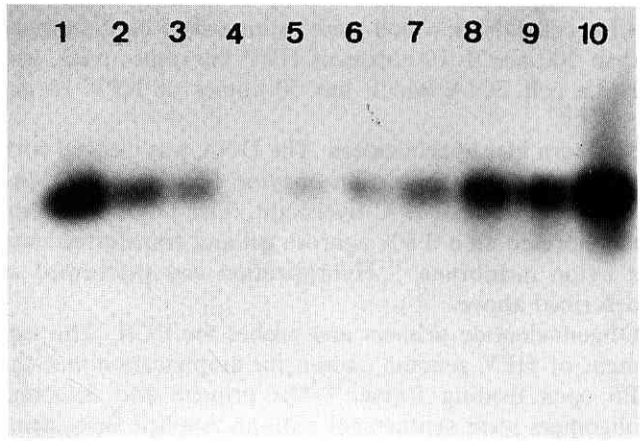


Fig. 2. Determination of PCR efficiency. CaSki DNA amplified for 30 cycles was loaded onto a gel along with purified 110-base fragments of target HPV 16 DNA, calculated to represent PCR efficiencies of the HPV 16 target sequence of 50, 55, 60, 65, 70 and 75%. (Lanes 1–3) 1.0, 0.2 and 0.1 $\mu$g of CaSki DNA; (lanes 5–10) $2\times10^{-2}$, $5.3\times10^{-2}$, $1.37\times10^{-1}$, $3.44\times10^{-1}$, $8.4\times10^{-1}$ and 2.0 pmol of purified 110-base fragment of HPV 16 DNA.

and 18 DNAs. The results obtained were in agreement with those by dot blot hybridization (Table I).

The 5 specimens containing both HPV 16 and 18 DNAs by dot blot were digested with *Pst*I or *Bam*HI and analyzed by Southern blot hybridization, first for HPV 16 DNA and then for HPV 18 DNA. The restriction profiles of 2 such specimens are shown in Fig. 1. HPV 16 has only 1 restriction site for *Bam*HI[5] and specimens containing HPV 16 should yield a single 8 kb band when digested with *Bam*HI and hybridized to HPV 16. However, in Fig. 1 (lanes 2 and 4), bands larger and smaller than 8 kb were obtained, implying that the HPV sequences were integrated. When the same specimens were hybridized to HPV 18, a restriction profile similar to that obtained for HeLa DNA was obtained.

**PCR amplification** The efficiency of the PCR was estimated using CaSki DNA. After 30 cycles of amplifica-

tion, the target sequence in 1 $\mu$g of CaSki DNA was amplified 1.3 million times (Fig. 2).

The primers and probes used in the PCR were specific for HPV sequences and no amplification was detected in the 2 cell lines containing Epstein-Barr virus DNA (Fig. 3, lanes 5 and 6) or in the water controls (lane 1). PCR amplification of a specimen containing HPV sequences resulted in a 110-base band on the gel. Amplification of DNA lacking HPV 16 and 18 sequences yielded either a faint random smear of polymerization or no band at the 110-base region (data not shown). The identities of the amplified products were further confirmed by hybridization with the oligomers H4 and H5 (Fig. 3). The oligomer H4 hybridized only to amplification products of CaSki and SiHa (HPV 16) primed with H1 and H2. No hybridization was seen when the primers H1 and H3 were used nor was there any between H4 and HeLa amplification products. Similarly, H5 only hybridized with HeLa amplified DNA (HPV 18) but not with CaSki or SiHa DNA.

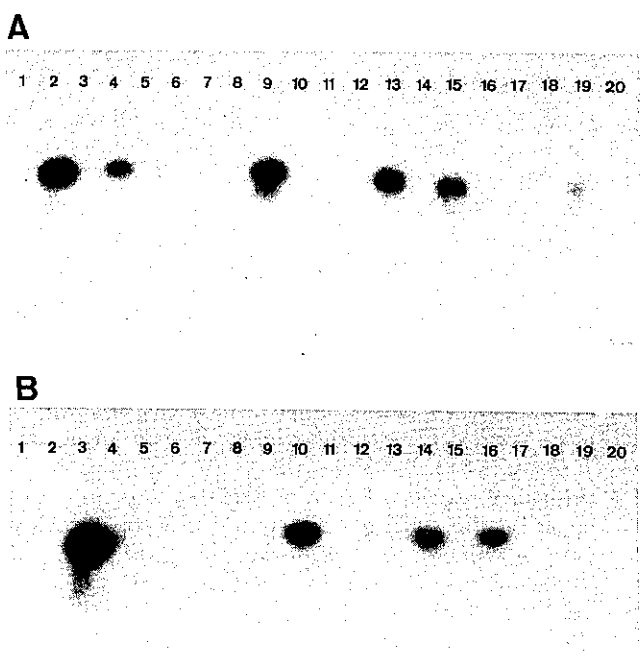


Fig. 3. Autoradiogram of PCR-amplified HPV sequences from 7 cervical cancers (lanes 7–20). Each specimen was probed with 2 sets of primers for HPV 16 and 18 sequences. Hybridization was done with (A) H4 specific for HPV 16 DNA; (B) H5 for HPV 18. Biopsied DNA (1 $\mu$g) was amplified with primers H1 and H2 (lanes 7, 9, 11, 13, 15, 17 and 19) and primers H1 and H3 (lanes 8, 10, 12, 14, 16, 18, and 20). Positive controls; CaSki, HeLa and SiHa (lanes 2–4). Negative controls; water, P3HR-1 and Raji (lanes 1, 5 and 6).

Of 23 specimens negative for HPV DNA by dot blot hybridization, 21 contained sufficient DNA for analysis by PCR. When the PCR amplification products were analyzed by gel electrophoresis alone, 13 were poitive for HPV sequences, with a 110-base fragment being visible on the gel after staining with ethidium bromide. However, after the amplification products were transferred onto a nylon membrane and probed with radioactively labeled detector oligomers, 4 more specimens were revealed to contain HPV DNA sequences. Thus, of 17 specimens positive for HPV sequences, 13 contained HPV 16 sequences, while 4 contained both HPV 16 and 18 sequences (Table I).

## DISCUSSION

The dot blot hybridization has been used by a number of investigators to detect HPV DNA in cervical biopsy specimens.[28–31] This technique is generally considered to be less sensitive and specific than Southern blot hybridization.[32, 33] We have used both hybridization techniques with similar results on HPV DNA-positive and negative biopsied specimens.

Studies carried out by laboratories in many different countries on the prevalence of HPV types in cervical cancers have shown great geographical variation. This might indicate an actual difference in the prevalence of HPV worldwide, or differences in sensitivity and specificity of the hybridization techniques used.[33] Findings by Brandsma *et al.*[34] have shown that identical specimens sent to 4 different experienced laboratories for the detection of HPV by Southern blot hybridization yielded significantly different results when their findings were compared. Hence, earlier epidemiological studies with a small number of samples and less sensitive methods of virus detection such as dot or Southern blot and filter *in situ* hybridization may have underestimated the true incidence of HPV. De Villiers *et al.* have acknowledged the insensitivity of the filter *in situ* hybridization technique used in the screening of 9,295 women in Germany and estimated an under-reporting by a factor of 2 to 3.[35]

Recently, the highly sensitive PCR technique has been used to improve the rate of detection of HPV DNA in women. With this technique, the incidence of HPV infection was found to range from 90% to 100% in biopsied specimens[16, 18] and 84% in normal swabs.[18] Morris *et al.*[36] screened for the presence of HPV 6, 11, 16, 18 and 33 in cervical lavage of 107 women attending a sexually transmitted diseases clinic by PCR and found 57 (53.3%) positive for HPV DNA. A similar study was also carried out by Shimada *et al.*[37] using Southern blot hybridization and PCR for the detection of HPV types 16, 18 and 33, in 43 cervical carcinomas. By Southern blot hybridization, they found HPV DNA in 60.4%

(26/43) of the patients. However, with PCR, the figure was increased to 83.7% (36/43). In our study, 95.1% (77/81) of specimens from invasive cervical cancer were found to contain HPV DNA sequences after PCR amplification, as compared with 72.3% (60/83) by Southern and/or dot blot hybridization.

Due to the extreme sensitivity of PCR DNA amplification, appropriate positive and negative controls were included with every assay and subjected to the same experimental conditions as for test specimens. The positive controls used were CaSki or SiHa and HeLa cell DNA. Under the conditions of the PCR, the 2 sets of primers were specific for their target sequences (H1 and H2 for HPV 16 and H1 and H3 for HPV 18) and did not amplify other HPV types. The detecting oligomers were also specific and H4 only hybridized to CaSki or SiHa amplification products, whilst H5 hybridized only to those of HeLa.

The use of radiolabeled probes in hybridization also increased the sensitivity and specificity of the PCR technique. It is possible that the PCR primers, by coincidence, can recognize homologous sequences in genomic DNA and, following amplification, produce a visible band on the gel which can only be distinguished from the actual target sequence by hybridization with the appropriate detecting oligomer.[38] Hybridization also increases the sensitivity by detecting small amounts of amplified sequences which are too faint to be visible on a gel.[39] In our study, 4 specimens undetectable by gel electrophorsis were found to be positive for HPV sequences after hybridization.

Despite the use of PCR, 4 specimens remained negative for HPV DNA. These may contain HPV types other than 16 or 18, or multiple sets of primers may be required to amplify different highly conserved regions of the HPV genomes. In our study, cross-reactivity with HPV 31, 33, 35, 52 and other HPV DNA was not determined. Shibata *et al.*[40] found that 2 sets of oligomers specific for 2 different cytomegalovirus (CMV) genes were required to detect all of the 28 wild-type CMV isolates.

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