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# Polymerase Chain Reaction Detection of Human Papillomavirus: Quantitation May Improve Clinical Utility

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**A case-control study compared detection by polymerase chain reaction (PCR) specific for human papillomavirus (HPV) type 16 with restriction enzyme analysis and Southern blot hybridization detection of HPV type 16. Cervicovaginal lavage samples from 64 women with histopathologic evidence of a cervical squamous intraepithelial lesion and 55 samples from cytologically healthy women were studied. Several methods of PCR product analysis, including radioactive and nonradioactive probing, were compared. The sensitivity of HPV detection by PCR when the amplified DNA fragment was visualized on a gel was equivalent to those of detection by restriction enzyme and Southern blot analyses. Hybridization of the PCR product with radioactively or nonradioactively labeled oligonucleotide probes increased the sensitivity of HPV detection by 100-fold. However, an increase in the sensitivity of the assay preferentially identified low levels of the virus in cytologically healthy women. Therefore, the value of HPV detection in identifying women with cervical neoplastic disease was greater, and the odds ratio for the presence of a cervical squamous intraepithelial lesion was higher when the less sensitive modalities were used. These results suggest that quantitation of HPV by PCR may maximize the clinical significance of a positive test result. Further studies will be needed to determine the optimal level of virus detection which has the highest positive predictive value of clinical disease.**

Considerable evidence implicates human papillomavirus (HPV) infection of the cervix as an important etiologic factor in the development of cervical neoplasia and cancer (8). Although mounting data indicate that HPV infection represents a major risk factor for the development of cervical neoplasia, it is also appreciated that other factors contribute independently to the development of squamous intraepithelial lesions (SILs) and cervical cancer (10, 11). Furthermore, HPV DNA has been detected in the lower genital tracts of many cytologically healthy women.

The reported prevalence of HPV infection has varied markedly in different populations and is greatly influenced by the methodology used to detect the virus. Southern blot hybridization has been considered the "gold standard" for HPV detection because of the test's ability to detect a wide range of HPV types, to eliminate false-positive signals, and to reveal information about the physical state of the viral DNA within the cell (12). The sensitivity of Southern blot hybridization is approximately 0.1 copy of HPV per cell when 10 μg of cellular ($10^6$ cells) DNA is assayed (12). Unfortunately, Southern blot hybridization is a time-consuming and complex procedure that is not well suited to most diagnostic laboratories.

The recent development of the polymerase chain reaction (PCR), an in vitro method of primer-directed enzymatic amplification of specific DNA sequences, has afforded greatly increased sensitivity, allowing detection of only a few copies of the HPV genome in an amplification reaction, or approximately 1 genome of HPV per $10^5$ cells. Furthermore, this method is technically easier, is less time-consuming, can be performed without radioisotopes, and yields results which do not require extensive interpretation. Experience in our laboratory suggests that typing by PCR may be more objective than that by Southern blot hybridization (data not shown).

However, significant variability in the prevalence of HPV infection has been noted between different studies that used PCR (1, 13, 15). Much of this variability may be accounted for by early problems with sample contamination, differences in amplification techniques, the viral types chosen for amplification, and the methods used to analyze the amplified product.

The clinical significance of detecting low-copy-number HPV DNA is largely unknown. Furthermore, several recent studies that used filter-in situ hybridization (11) or Southern blotting and PCR (10) have suggested that the risk of cervical neoplasia is greatest in patients with quantitatively more virus present in their cervicovaginal cells.

HPV type 16 (HPV-16) is the papillomavirus type which has been most commonly associated with cervical neoplasia (3). Therefore, we designed oligonucleotide primers specific for HPV-16 DNA amplification and examined several methods of PCR product analysis, including radioactive and nonradioactive hybridization techniques, in order to evaluate the relationship between test sensitivity and the association of HPV-16 with cervical neoplasia in a case-control study.

## MATERIALS AND METHODS

**Study population and specimen collection.** The study protocol was approved by the hospital Institution Review Board, and informed consent was obtained from subjects prior to enrollment in the study. The subjects included in this analysis represented a subset of subjects in a larger study which evaluated the role of HPV infection in the development of SILs (10). Patients were women referred to the colposcopy clinic of a large, urban, municipal hospital for evaluation of recent abnormal Papanicolaou (Pap) smears.

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Evaluation of these women included a gynecologic examination with a Pap smear, cervicovaginal lavage (CVL) (6), colposcopic examination, and colposcopically directed cervical biopsy. A patient was defined as a nonpregnant woman with an SIL on cervical biopsy and adequate DNA obtained via CVL for both Southern blot and PCR testing for HPV. Sixty-four subjects met these criteria. SIL lesions included koilocytotic atypia ($n$ = 6), cervical intraepithelial neoplasia stage I (CIN I) ($n$ = 19), CIN II ($n$ = 18), and CIN III ($n$ = 21).

Control subjects from family planning and gynecology clinics of the same hospital were enrolled in the study. Patient evaluations included a gynecological examination with a Pap smear and CVL. A control was defined as a nonpregnant patient who had negative cervical cytology and a CVL sample which contained adequate DNA for both Southern blot and PCR analyses. Fifty-five subjects met these criteria.

**Southern blot hybridization.** DNA was obtained from CVL samples by digestion with proteinase K, phenol-chloroform extraction, and ethanol precipitation. DNA was digested with *Pst*I, separated by electrophoresis in an agarose gel, and transferred to a nylon filter. Hybridization was performed by using a mixed probe of $^{32}$P-labeled HPV-11, -16, and -18 DNAs. Filters were washed under low-stringency conditions ($T_m$ − 40°C); this was followed by autoradiography for periods of up to 36 h. Filters were then rewashed under conditions of high stringency ($T_m$ − 10°C) and were reexposed for periods of up to 14 days. HPV-16 was identified from the specific *Pst*I restriction enzyme cleavage pattern present under stringent conditions. Southern blot interpretations were made by a single, experienced observer who did not have knowledge of clinical or cytologic findings.

**PCR.** PCR was done by using HPV-16-specific primers which amplified a 246-bp region from nucleotides 5939 to 6184 in the L1 open reading frame. The positive-strand primer sequence was 5′-GCCTGTGTAGGTGTTGAGGT-3′, and the negative-strand primer sequence was 5′-TG GATTTACTGCAACATTGG-3′. These primers flanked the plasmid cloning site, eliminating potential contamination from cloned HPV-16. Concentration of reagents and reaction conditions were optimized to achieve maximal sensitivity in the detection of HPV-16 (7). Sensitivity was determined by the amplification of DNA extracted from SiHa cells by serially diluting the DNA into HPV-negative human genomic DNA. PCR mixtures consisted of 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 3.5 mM $MgCl_2$; 0.01% gelatin; 200 μM (each) dATP, dCTP, dGTP, and dTTP; 1.0 μM (each) primer; 1.25 U of Amplitaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.); and 1.5 to 3.0 μg of genomic DNA per 50-μl reaction covered with mineral oil. Reagents were mixed in an area free from PCR products, and extreme care was used to avoid contamination. Appropriate positive (SiHa DNA) and negative (reagents only and HPV-negative DNA) controls were included for each 15 samples. Thirty cycles of amplification each consisted of 1 min of denaturation at 94°C, 2 min of primer annealing at 60°C, and 3 min of primer extension at 72°C in a thermocycler (Perkin-Elmer Cetus).

PCR products were analyzed by the following three methods: (i) electrophoresis in a 3% NuSieve–0.5% agarose gel (FMC, Rockland, Maine) followed by observation of the appropriately sized 246-bp DNA fragment after ethidium bromide staining and UV fluorescence; (ii) Southern transfer to a nylon filter, which was then probed with a $^{32}$P-end-labeled 20-bp internal probe; and (iii) elution of the probe from the filter followed by reprobing by using the same internal probe nonradioactively labeled with digoxigenin-11-dUTP (Genius; Boehringer, Mannheim, Germany).

The internal probe corresponded to HPV-16 nucleotide 6039 to 6058 sequences, 5′-TATGCAGCAAATGCAGG TGT-3′. The probe was end-labeled with polynucleotide kinase and [γ-$^{32}$P]ATP. The filters were hybridized overnight at 42°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–20 mM $NaPO_4$–0.1% sodium dodecyl sulfate (SDS)–10× Denhardt solution; they were then washed at 48°C. The final wash was done in 1× SSC–1% SDS at 48°C. Autoradiography for periods of up to 24 h was followed by elution of the probe from the filters in 1× SSC at 78°C and repeat autoradiography to verify that the probe had been removed.

Digoxigenin-11-dUTP and dATP were used to label the nonradioactive internal oligonucleotide probe as described in the Boehringer tailing kit. Filters were prehybridized in 5× SSC–0.5% blocking reagent (Boehringer)–0.1% *N*-lauroylsarcosine, sodium salt–0.02% SDS for a minimum of 1 h and were then hybridized overnight at 42°C. Filters were washed with 1.5× SSC–0.1% SDS at 48°C as the final wash. Incubation with anti-digoxigenin antibody conjugated to alkaline phosphatase, subsequent washes, and overnight incubation in nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt in dimethylformamide in an alkaline buffer were performed as described for the Genius protocol (Boehringer). PCR product analyses were interpreted without knowledge of cytologic or pathologic findings, and results were then tabulated for comparison of the different methodologies.

## RESULTS

The amplification of DNA extracted from SiHa cells and serially diluted into HPV-negative human genomic DNA revealed that the 246-bp DNA fragment was inadequately visualized in the gel by UV fluorescence when the initial reaction contained 50.0 ng of SiHa DNA in 1.0 μg of total DNA. Since SiHa cells have one to two integrated copies of HPV-16 per cell (4) and a single cell contains approximately 7 pg of DNA, 50.0 ng of SiHa DNA contains close to 7,000 copies of HPV-16. Hybridization of the PCR product with the internal oligonucleotide probes increased the sensitivity of HPV-16 detection 100-fold. The amplified DNA fragment was consistently identified from reactions which contained 0.5 ng of SiHa DNA, but it was not apparent in reactions containing 0.05 ng of SiHa DNA. Therefore, the lower limit of detection of HPV-16 by PCR followed by hybridization with the oligonucleotide probe was between 7 and 70 copies of HPV-16 per reaction. There was no difference in sensitivity when the radioactive or nonradioactive probes were used (Fig. 1).

Table 1 shows the prevalence of HPV-16 in controls and cases as detected by Southern blot hybridization of cervicovaginal cell DNA or by PCR followed by different methods of PCR product analysis. The sensitivity of HPV-16 detection by Southern blot analysis was comparable to that by PCR amplification followed by ethidium bromide staining and UV fluorescence. HPV-16 was detected by Southern blot analysis in 19% of cases and 4% of controls. PCR amplification followed by ethidium bromide staining and UV fluorescence detected HPV-16 in 25% of cases and 4% of controls.

PCR followed by visualization of the band in the ethidium bromide-stained gel did not identify four Southern blot-positive subjects. Likewise, PCR-ethidium bromide staining

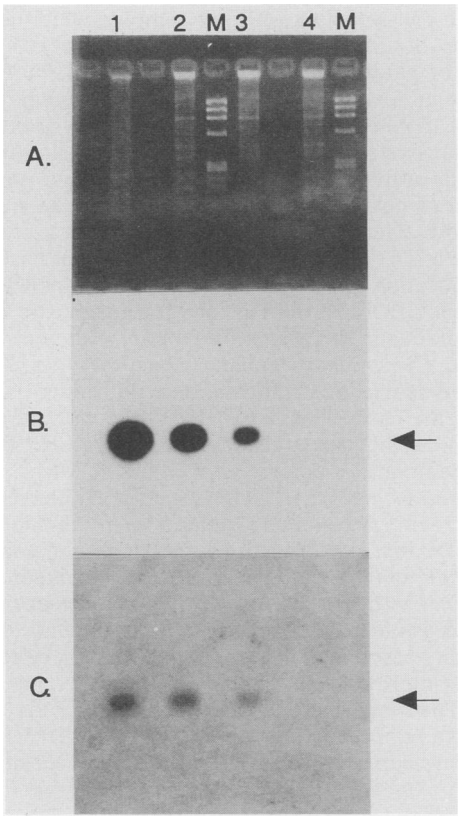


FIG. 1. Detection of HPV-16 PCR products. SiHa DNA was serially diluted into human genomic DNA and amplified with HPV-16-specific primers as described in the text. Aliquots of 20 μl of the PCR products from reactions containing 50.0, 5.0, 0.5, and 0.05 ng of SiHa DNA are shown in lanes 1 through 4, respectively. (A) PCR products in the gel after electrophoresis in a 3% NuSieve–0.5% SeaKem agarose gel, ethidium bromide staining, and UV fluorescence. Following Southern transfer, the PCR products were hybridized with a $^{32}$P-end-labeled HPV-16 oligonucleotide probe, as shown in the autoradiogram (B). The filter was then stripped and reprobed with a digoxigenin-11-dUTP-labeled HPV-16 oligonucleotide probe. Use of the Genius colorimetric detection system revealed hybridization to the PCR product (C). Arrows indicate the presence of the 246-bp HPV-16 DNA-hybridizing fragment. Lane M (marker), ϕX174 DNA digested with *Hae*III.

identified eight subjects who were not positive by Southern blot hybridization. A kappa coefficient was calculated to assess the degree to which agreement between the PCR-ethidium bromide staining and direct Southern blot results exceeded chance agreement. A kappa coefficient of 1.0 indicates complete agreement between two tests, and a value of 0 indicates agreement consistent with chance. The overall agreement between PCR-ethidium bromide staining and Southern blot results was highly correlated ($P < 0.01$). However, the calculated kappa coefficient of 0.57 suggests only fair to good agreement in identifying the same subjects by the different tests.

All subjects positive by PCR-ethidium bromide staining or Southern blot analysis were verified as positive by PCR followed by hybridization with the oligonucleotide probe. Probing of the amplified products with the $^{32}$P-labeled probe identified the HPV-16 DNA fragments in 50% of cases and 22% of controls. The nonradioactive (Genius) probe identified the HPV-16 fragments in 55% of cases and 22% of controls. The kappa coefficient, 0.88, demonstrated excellent agreement between the $^{32}$P-labeled probe and nonradioactive probe results.

TABLE 1. Results of HPV-16 detection by different methods in patients classified by cytologic or histopathologic diagnoses

| Method | HPV-16 prevalence in the following subjects (no. [%]): | | | |
|---|---|---|---|---|
| | Controls ($n$ = 55) | KA/CIN stage I ($n$ = 25)[a] | CIN stage II ($n$ = 18) | CIN stage III ($n$ = 21) |
| Southern blotting | 2 (4) | 1 (4) | 5 (28) | 6 (29) |
| PCR | | | | |
| Ethidium bromide staining, UV fluorescence | 2 (4) | 2 (8) | 6 (33) | 8 (38) |
| $^{32}$P-labeled probe | 12 (22) | 10 (40) | 9 (50) | 13 (62) |
| Genius probe | 12 (22) | 12 (48) | 10 (55) | 13 (62) |

[a] KA, koilocytotic atypia; CIN, cervical intraepithelial neoplasia.

The reproducibility of HPV test results has been excellent in our laboratory. Repeated PCR of 28 blinded samples from this study yielded identical results for 27 (96%) samples. Past assessment of reproducibility of HPV test results by Southern blot hybridization for the detection of a wide variety of HPV types yielded agreement on 19 of 20 (95%) samples.

Analyses were performed to investigate the association of HPV-16 with SILs. Odds ratios serve as an approximation of the increased risk of SILs associated with the detection of HPV-16. PCR followed by probing with $^{32}$P or the Genius probe yielded odds ratios of 3.6 and 4.3, respectively (Table 2). The odds ratios were higher when Southern blotting (odds ratio, 6.1) or PCR with gel visualization (odds ratio, 8.8) was used. Therefore, the risk of SIL associated with detection of HPV-16 was greater when the less sensitive detection techniques were used. In addition, the specificity of the test was also greater when a less sensitive methodology was used.

In order to assess the clinical utility of HPV-16 detection as a general screening test for cervical dysplasia, we calcu-

TABLE 2. Results of HPV-16 detection by different methods

| Method | Prevalence (no. of subjects [%]) in: | | Odds ratio[a] | Specificity (%)[b] | Positive predictive value (%)[c] |
|---|---|---|---|---|---|
| | Controls ($n$ = 55) | Cases ($n$ = 64) | | | |
| Southern blot | 2 (4) | 12 (19) | 6.1 | 96 | 20 |
| PCR | | | | | |
| Ethidium bromide staining, UV fluorescence | 2 (4) | 16 (25) | 8.8 | 96 | 25 |
| $^{32}$P-labeled probe | 12 (22) | 32 (50) | 3.6 | 78 | 11 |
| Genius probe | 12 (22) | 35 (55) | 4.3 | 78 | 12 |

[a] Odds ratios are an approximation of the increased risk of cervical dysplasia associated with the detection of HPV-16.

[b] Specificity refers to the probability that a patient without cervical dysplasia will test negative for HPV-16.

[c] Positive predictive value indicates the proportion of positive tests that identify individuals with disease and is calculated on the basis of an assumed population prevalence of cervical dysplasia of 5%.

lated the predictive value of a positive test. A 5% prevalence of cervical cytologic abnormalities was assumed and used in the calculations (Table 2). An increase in the sensitivity of virus detection decreased the predictive value of the test.

Because the PCR-ethidium bromide staining and Southern blot methodologies were not totally concordant, the results of these tests were combined such that subjects positive by either or both tests were considered to be HPV-16 positive. This more complex testing strategy yielded a somewhat higher odds ratio (12.0) and a greater positive predictive value (31%).

## DISCUSSION

Discrepancies in the prevalence of PCR-detected HPV in previous studies may be related to methodologic variables. Primers (13), reaction conditions, cycling parameters, and magnesium concentration have been observed to affect the reaction efficiency (7). Previous studies have also used a variety of methods to confirm the presence of the amplified DNA fragment in the PCR product, as follows: electrophoresis in agarose or acrylamide gels with ethidium bromide staining and UV fluorescence, restriction enzyme analysis of the PCR product, sequencing of the PCR product, or hybridization with DNA probes after Southern transfer or dot blot hybridization.

The results of this study demonstrate that the method of product analysis greatly affects the sensitivity and specificity of the assay. Indeed, hybridization with a DNA probe increased the sensitivity of HPV-16 detection by a factor of 100, which translated into a 2.2-fold increase in the perceived viral prevalence in the cases and a 5.5-fold increase in the rate of viral detection in the controls. An increase in the sensitivity of the assay appeared to preferentially identify low-level or latent HPV-16 infections in cytologically healthy women. Therefore, the odds ratios for the presence of cervical abnormalities were higher and the positive predictive values were greater when the less sensitive detection modalities were used.

These results are similar to those noted in a comparison of Virapap/Viratype with PCR specific for HPV types 6, 11, 16, and 18. Burmer et al. (2) suggested that PCR is a clinically useful methodology for screening high-risk populations. However, their data showed a 3.1-fold increase in the prevalence of HPV detected in cytologically healthy women, in contrast to only a 1.4-fold increase in the prevalence of HPV detected in women with dysplasias, when PCR was compared with Virapap. PCR again preferentially increased the identification of virus in cytologically healthy women. Therefore, the predictive value of a positive PCR was lower than the predictive value of a positive Virapap.

These findings are not surprising in light of recent observations in our population (10) and those of Reeves et al. (11), which suggested that the risk of cervical neoplasia was greatest in women with quantitatively higher viral loads.

PCR can be used in a quantitative manner (9). Unfortunately, the complexity of this technique may limit its usefulness. However, a simple semiquantitative estimate of viral load based solely on the signal strength of the probe that hybridizes to the PCR product demonstrated that increased signal strength was associated with an increased risk of SIL (10) when these or other type-specific PCR primers were used. The applicability of this approach to other PCR primers or reaction conditions remains to be demonstrated.

The use of PCR for research purposes has blossomed. Experience with PCR suggests that this is an important technique for detecting HPV in samples which are inadequate for Southern blotting (samples with little cellular material or paraffin-embedded samples) (14). Furthermore, PCR may be technically easier to perform and may provide an easier, more objective method of viral typing than Southern blot analysis. Finally, the increased sensitivity of PCR may be desirable in studies in which the end point is the detection of virus, allowing an estimate of the true prevalence and incidence of HPV infection and an assessment of the association between HPV and its related disease. Hence, misclassification of HPV status should be minimized (5).

However, PCR is moving into the diagnostic arena. In clinical practice, there is no effective treatment for HPV infection. The clinical significance of HPV centers on the associated neoplastic manifestations and cancer risk. Unfortunately, the natural history of HPV infection has not been clarified, and the cancer risk associated with HPV-16 infection in cytologically healthy women is unknown.

At this time, use of HPV testing as a supplement to Pap smear screening is limited by the poor predictive value of virus detection in populations in which the majority of women are cytologically healthy. Selective screening of populations with risk factors for cervical cancer or individuals, such as older women, in which the presence of HPV is more highly associated with the presence of cervical disease (10) will improve the positive predictive value of HPV testing. The results of our study demonstrate that development of a clinically relevant PCR-based HPV test will depend on determination of the optimal test sensitivity.

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