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Comparison of Dot Filter Hybridization, Southern Transfer Hybridization, and Polymerase Chain Reaction Amplification for Diagnosis of Anal Human Papillomavirus Infection

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The detection and classification of human papillomavirus (HPV) by a consensus primer polymerase chain reaction (PCR) technique were compared with detection and classification by dot filter hybridization (DFH) and Southern transfer hybridization (STH). PCR detected HPV in 87% of specimens; the detection rates for DFH and STH were 51% and 49%, respectively. The specific HPV types detected by STH were also detected by PCR in 90% of specimens. However, 75% of the samples positive for unclassified HPV by STH were typed by PCR. PCR results were reproducible, as assessed by repeat analysis (96% agreement), by analysis of paired same-day specimens (89% agreement), and by interlaboratory analysis (88% agreement). PCR is a sensitive, specific, and reproducible test for HPV detection and classification in clinical and epidemiologic studies.

Human papillomavirus (HPV) is a common sexually transmitted pathogen that is now thought to play an important role in the pathogenesis of genital cancer (10, 21). The sensitive, specific, and reproducible identification of HPV in clinical samples is essential if we are to understand the natural history and the role of specific types of HPV in various neoplasias. Since the virus cannot be cultured, detection of specific types of HPV has been based on DNA hybridization tests, including Southern transfer hybridization (STH) and dot filter hybridization (DFH), which previously have been shown to have equivalent sensitivity, specificity, and reproducibility (1, 7, 11, 13, 14). Amplification of HPV DNA by a consensus primer polymerase chain reaction (PCR) technique before hybridization has offered increased sensitivity (3). However, little is known about the specificity and reproducibility of this assay with clinical samples in comparison with nonamplified hybridization assays.

To address these issues, we analyzed by STH, DFH, and PCR 893 anal epithelial cell samples from 443 homosexual men enrolled in a study of anal squamous intraepithelial lesions at the Seattle-King County Department of Public Health AIDS Prevention Clinic (12). The reproducibility of PCR was assessed by analysis of duplicate samples obtained from a subset of consecutive patients, by subsequent retesting of samples, and by analyzing samples independently in two laboratories. Informed consent was obtained from all patients, and all procedures were approved by the Human Subjects Committees of the University of Washington and the Seattle-King County Department of Public Health.

HPV DNA detection methods. Specimens for anal HPV testing were collected on Dacron swabs and placed in HPV specimen transport medium (Digene Diagnostics, Silver Spring, Md.) (12). For DFH, 250 μl of each sample was analyzed for HPV types 6, 11, 16, 18, 31, 33, and 35 with a commercial kit (ViraPap; Digene) according to the manufacturer's specifications. This assay does not include any method of determining specimen adequacy, and thus all results were included.

For STH, 250 μl of each sample was analyzed by low-stringency hybridization for any HPV and by high-stringency hybridization for HPV types 6 or 11, 16 or 18, and 31, 33, or 35 as previously described (13). HPV DNA for the controls and probes was purified from HPV plasmids (13). Samples negative for DNA by ethidium bromide staining (n = 44) and samples giving positive autoradiographic signals but smeared banding patterns (n = 34) were classified as unsatisfactory (9%). Samples hybridizing at low stringency but not to any probe mixture at high stringency were considered positive for unclassified HPV.

For PCR, 20 μl of each sample was analyzed in duplicate as previously described (2, 3). Briefly, two sets of commercially available primers (Perkin-Elmer, Norwalk, Conn.) were used; consensus primers MY09 and MY11 target an approximately 450-bp region of the HPV L1 gene, and PC04 and GH20 target a 268-bp region of the human β-globin gene (3). The presence of the β-globin fragment on ethidium bromide-stained gels after electrophoresis of the PCR products was used to assess whether the samples contained adequate DNA and whether PCR inhibitors were present. PCR samples negative for the β-globin fragment (21%) were considered unsatisfactory. After amplification, 3 μl of each product was spotted onto filters and hybridized with a biotin-labeled generic HPV probe (2) and with mixtures of biotin-labeled type-specific oligonucleotide probes for HPV types 6 or 11, 16 or 18, and 31, 33, or 35 (2). Samples hybridizing with the generic probe but not with any of the type-specific probes were considered positive for unclassified HPV.

Each PCR run included the following controls: (i) DNA from 4×10^2 SiHa cells, an HPV type 16-positive cell line; (ii) DNA from K562 cells, an HPV-negative human cell line; (iii) K562 cells in specimen transport medium (10^6/ml), handled concurrently with the patient samples; and (iv) a

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TABLE 1. Detection of HPV DNA in 893 anal epithelial cell samples from 443 homosexual men by PCR amplification, DFH, and STH

PCR result[a]	DFH result[b]	No. of samples with following STH result[c]:			Total
		Negative	Positive	Unsatisfactory	
Negative	Negative	78	1	6	85
	Positive	3	0	0	3
	Total	81	1	6	88
Positive	Negative	200	37	28	265
	Positive	46	282	25	353
	Total	246	319	53	618
Unsatisfactory	Negative	131	6	18	155
	Positive	9	22	1	32
	Total	140	28	19	187
Total		467	348	78	893

[a] PCR amplification with consensus primers directed to the HPV L1 gene followed by dot filter hybridization with a generic probe and type-specific oligonucleotide probes.
[b] ViraPap HPV DNA detection kit (Digene).
[c] At low and high stringencies.

reagent blank (no DNA). Environmental controls for PCR were obtained by swabbing the upper backs of 139 of the 443 patients and were analyzed concurrently with the anal swabs. Two back samples were positive for unclassified HPV, and one was positive for HPV type 6 or 11. Although these three samples may have been falsely positive because of contamination, they represented only a small percentage (2%) of the back samples analyzed. All other negative controls for all three methods were negative for HPV DNA.

HPV detection and type classification. Of the 647 samples that were satisfactory by all three methods, 565 (87%), 331 (51%), and 320 (49%) were HPV DNA positive by PCR, DFH, and STH, respectively (Table 1). HPV DNA was detected by all three methods in 282 (44%) samples and was undetectable by all three tests in 78 (12%). Eighty-three (13%) samples were positive by PCR and either STH or DFH, 200 (31%) were positive only by PCR, 3 were positive only by DFH, and 1 was positive only by STH. Almost all discrepancies were cases reflecting the expected increased sensitivity of PCR.

The specific HPV types detected by STH were also detected by PCR in 220 (90%) of the 244 samples that were typed by STH (Table 2). Review of the STH autoradiographs and reanalysis by PCR of the 24 discrepant samples confirmed the original results. The HPV types identified by STH but not by PCR were equally distributed among the three probe groups (data not shown). The weighted kappa statistic (19) assessing agreement between detection of specific HPV types by PCR and detection of these types by STH was 0.41, reflecting the tendency of PCR to detect additional HPV types not detected by STH and the high percentage of positive samples from this high-risk population. The high proportion of samples positive by PCR for more than one HPV type (59%) is also a reflection of this population at high risk for anal HPV infection and has been reported previously (6).

TABLE 2. Comparison of specific HPV types detected by STH and PCR in 319 anal epithelial cell samples found positive by both methods

HPV type detected by STH	No. of samples in which indicated type was detected by PCR				
	Unclassified	6 or 11	31, 33, or 35	16 or 18	Multiple
Unidentified	19	11	8	9	28
6 or 11	1	32	0	1	38
31, 33, or 35	1	0	9	4	16
16 or 18	4	1	0	25	39
Multiple	0	3	0	4	66

While the specificity of the PCR method was equal to that of STH for the identification of the seven HPV types used as specific probes, PCR was better than STH at detecting the unclassified types of HPV. Fifty-six (75%) of the 75 samples positive for unclassified HPV by STH were typed by PCR, while only 6 (24%) of the 25 samples positive for unclassified HPV by PCR were typed by STH (Table 2). This may be explained by the fact that for STH, low-stringency hybridization was performed first, and then the filters were stripped and reprobed for each subsequent type-specific hybridization. As a result, small amounts of HPV DNA giving a positive signal on the first hybridization may have been removed and not detected on later hybridizations.

Two hundred forty-six samples were positive by PCR and negative by STH. As has been previously reported for cervical lavage samples (18), a larger proportion of the PCR-positive, STH-negative anal samples (43%) contained only unclassified HPV DNA than did the samples that were positive by both methods (8%). The PCR generic probe probably detected a broader spectrum of genital HPV types than did the low-stringency STH technique. Furthermore, the unclassified HPV types may have been present in concentrations not detectable by STH. Alternatively, since the generic probe was two to five times more sensitive than the type-specific probes, specimens with extremely low levels of type-specific HPV DNA may have been positive by the generic probe only. To confirm that the PCR-positive, STH-negative samples containing only unclassified HPV were truly HPV positive, we analyzed the PCR amplification products using genomic HPV probes under low-stringency hybridization conditions. Seventy-nine (75%) of the 105 PCR products tested were positive.

Reproducibility of PCR. The reproducibility of PCR was assessed by subsequent retesting of samples and by analysis of duplicate samples. Among 80 samples reanalyzed several months after the initial analysis, there was complete agreement on detection and typing of HPV DNA for 77 (96%). In the three discrepant samples, at least one HPV type was detected in both analyses, with an additional type identified in only one. These samples were considered partially discrepant. Of 35 duplicate swab samples, there was complete agreement on detection and typing of HPV DNA for 31 (89%). In two of the four discrepant pairs, HPV DNA was detected in only one sample; the other two pairs were partially discrepant.

Interlaboratory reproducibility was assessed by testing 100 samples in two laboratories. Ten samples were unsatisfactory in both laboratories, 17 were unsatisfactory in laboratory A only, and 3 were unsatisfactory in laboratory B only. In laboratory A, 12 of the unsatisfactory samples were from one run; however, a lack of additional sample precluded further PCR analysis. Of the 70 specimens satisfactory in both laboratories, 9 were negative and 49 were positive for HPV in both laboratories (83% agreement). Six of the 49 samples positive in both laboratories were partially discrepant (88% agreement). Of the 12 samples that were

TABLE 3. Comparison of PCR results for 207 samples from initial visits with results for 340 samples from follow-up visits.

Result	No. (%) of patients
Negative for HPV DNA on all visits	11 (5)
Same HPV type(s) detected on all visits	71 (34)
Same HPV type(s) detected on all visits, with an additional HPV type detected on ≥1 visit	97 (47)
HPV detected on 1 visit only	16 (8)
Different HPV type detected at each visit	12 (6)
Total	207 (100)

positive in one laboratory only, 7 were weakly positive for unclassified HPV and 1 was weakly positive for HPV type 6 or 11. The weighted kappa statistic assessing specific type agreement between laboratories was 0.70. Discordant results may have been due to differences between laboratories in interpretation of weakly positive signals or to the failure of a minute amount of HPV DNA to be amplified or detected.

To determine whether the type of HPV DNA identified by the PCR method could be consistently detected in samples obtained from the same patient over time, we compared the results of 547 samples obtained during the initial and follow-up visits of 207 patients (Table 3). The number of follow-up visits per patient ranged from one to five over a period of 1 week to 19 months. The results from samples obtained during initial and follow-up visits were exactly the same or only partially discrepant for 86% of the patients, indicating the consistency over time of the PCR results for this population of men at high risk for expression of HPV.

In this study we compared the sensitivity, specificity, and reproducibility of PCR with those of DFH and STH for the detection and classification of HPV. PCR was more sensitive than DFH or STH for the detection of HPV DNA in anal samples. For cervical specimens, other investigators have reported 1.2- to 7-fold higher prevalences of HPV DNA when PCR was used for detection than when DFH (3, 5, 7, 8, 16) or STH (15, 17, 18, 20) was used, although differences in study populations and HPV types detected by each method make comparisons difficult. We found the PCR method as specific as STH for the identification of seven HPV types and better for detecting unclassified types. The reproducibility of PCR was similar to that previously reported for STH (4).

A larger proportion of specimens were unsatisfactory for analysis by PCR (21%) than for STH (9%). Since only 7% of the cytology specimens were unsatisfactory because of an insufficient number of cells, we feel that the majority of unsatisfactory PCR samples were due to inhibition of amplification. Interestingly, only 6% of the cervical samples analyzed by PCR in our laboratory were unsatisfactory. Since none of the anal samples contained blood, a recognized PCR inhibitor (9), other unrecognized inhibitors may have been present in these samples. Twenty percent of the anal samples unsatisfactory by PCR were positive by STH and/or DFH, demonstrating the potential usefulness of employing more than one assay for HPV DNA analysis.

In summary, combined use of PCR and STH can give valuable information about the types and levels of HPV present in clinical samples. At present, the clinical relevance of samples that are positive by PCR and negative by unamplified methods is not clear. The most useful HPV assay is likely to be one test with high sensitivity that could also evaluate the levels of HPV DNA present in the samples.

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