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Evaluation of Methods for Detecting Human Papillomavirus Deoxyribonucleotide Sequences in Clinical Specimens

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Specimens from 26 condylomatous lesions, 24 invasive cancer cells, and 33 cervices, without evidence of the diseases, were tested for the presence of human papillomavirus (HPV) types 6, 11, 16, and 18 by Southern blot hybridization, in situ filter hybridization, or in situ tissue hybridization methods. A total of 89% (23 of 26) of the condylomatous lesions contained HPV DNAs, as determined by one or more of the methods. The positive rates for the detection of HPV DNA in condylomas by the different methods were 82% for Southern blot hybridization, 62% for in situ filter hybridization, and 72% for in situ tissue hybridization. Among the specimens from patients with cancer, HPV DNA was found in 83% (19 of 23) by one or more of the methods. Positive rates of 89 and 70%, respectively, were obtained for cancer lesions tested by the filter in situ and Southern blot hybridization methods; however, only 30% of those lesions were positive by the in situ tissue hybridization method. Thirteen percent of the control cervices were positive for HPV DNA by one or more of the assays. With respect to all disease categories, the methods had comparable sensitivities and specificities, except for the in situ tissue hybridization method, which revealed a specificity of 72% for condylomatous lesions and 30% for invasive cancer cells.

Papillomaviruses (PVs) belong to a subgroup of *Papovaviridae* which are characterized by a small, double-stranded, circular DNA genome (25). The PVs are species specific and exhibit a high degree of tissue tropisms. In humans, over 45 types of human PVs (HPVs) have been isolated from patients with such diverse clinical conditions as cutaneous warts, anogenital warts, laryngeal papillomas, and epidermodysplasia verruciformis (17). In addition, specific genotypes of HPV have been implicated as the cause of histopathological changes in cervical intraepithelial neoplasia (CIN) lesions and in the genesis of invasive cervical cancer (28). The prevalence of HPV types 11 and 6 has been reported to be between 85 and 100% in benign condylomas and between 18 and 30% in advanced CIN lesions (7, 9, 26). In contrast, HPV type 16 seems to prevail in more severe forms of CIN and invasive cervical cancers, with the reported prevalence rates varying from 17 to 100% in invasive cervical cancers (5, 6, 21, 28). HPV type 18 has also been associated with invasive cervical cancers, but its prevalence is lower than that of HPV type 16 (4, 28).

Progress in elucidating the epidemiology and pathogenesis of the HPVs has been hampered by the lack of suitable conventional laboratory techniques to culture the viruses in vitro. Vegetative replication of PVs is confined to the specific host and to the degree of differentiation of the host cells (16). Only the terminally differentiating keratinocytes of the particular host seem to be permissive to infection by PVs (17). Therefore, the diagnosis of PVs in clinical samples rests upon the presence of complete virions as shown by electron microscopy (1), the presence of virion structural antigens as shown by immunological methods (13), or detection of virus-specific DNA sequences by molecular hybridization methods.

The success of electron microscopy in the diagnosis of HPV depends on the demonstration of the HPV virions, while that of the immunological tests depends on the presence of the common PV structural antigens. Virion assembly and expression of the late structural antigens takes place only in terminally differentiated cells. Thus, both virions and antigens are absent in grade 3 CIN lesions, which, by definition, are undifferentiated (19). Consequently, the sensitivity of these tests in the diagnosis of HPV from these lesions ranges between 40 and 70% (17). Furthermore, neither assay is capable of distinguishing the subtypes of PV that are responsible for the infection. These limitations have been largely overcome by cloning the HPV DNA in bacterial plasmids, since this permits the development of viral probes which can be used in molecular hybridization assays to detect specific viral DNA sequences. Three such methods have been developed: Southern blot hybridization, in situ filter hybridization, and in situ tissue section hybridization. The purpose of this study was to define the relative sensitivities and specificities of these methods for detecting HPV DNA sequences in papillomatous lesions, cervical cancer cells, and cervices without evidence of the diseases.

MATERIALS AND METHODS

Study population and preparation of samples. Samples were obtained from three categories of patients from two different populations. The first category consisted of 26 Canadian patients with genital condylomas. These patients were seen at university-affiliated clinics in Hamilton, Ontario. We obtained specimens from 6 penile, 6 vulvar, 2 groin, and 12 cervico-vaginal lesions. The second category consisted of 23 Panamanian women with newly diagnosed cervical cancers. The third category consisted of 21 Canadian and 12 Panamanian patients who had undergone hysterectomy for diseases other than neoplasia. Histologic examination confirmed the diagnoses of condyloma or invasive cervical cancer in the first and second categories and the

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absence of these lesions in the third category. Although patients in the third category were considered normal for the purposes of this study, histologic examination revealed 9 patients with cervicitis, 11 patients with cervical metaplasia, and 13 patients with unremarkable cervices.

We attempted to collect cells from the lesion from each patient for in situ filter hybridization and then to obtain two biopsies of the lesions. This was not achieved in all cases. For the in situ filter hybridization method, cells from the cervical os or cervical lesions were collected with a cotton-tipped swab and suspended in 4 ml of phosphate-buffered saline. The number of cells was counted in a hemacytometer, and the cells were stored at −20°C until they were tested. The cell counts ranged from 10^4 to 10^7 epithelial cells per sample. Biopsies intended for the Southern blot method were snap-frozen in liquid nitrogen for transportation to the laboratory, where they were subsequently stored at −70°C until they were tested. Biopsies for the histopathological examination and in situ tissue hybridization methods were fixed in 10% buffered, neutral Formalin and processed by routine histologic methods.

For the in situ filter hybridization method, the samples were thawed, and the cells were pelleted at 2,000 × *g* for 10 min and suspended in 400 μl of a buffer consisting of 5 μg of denatured salmon sperm DNA per ml in 6× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate [pH 7.0]). The extraction of DNA from biopsies for Southern blot analysis was done as described previously (18) by using proteinase K digestion and phenol-chloroform extraction. Sections for use in the in situ tissue hybridization method were cut to a 5-μm thickness from paraffin-embedded blocks. The sections were placed on poly-D-lysine (Sigma Chemical Co., St. Louis, Mo.)-coated glass slides and fixed by baking the slides at 60°C overnight.

Probe DNA and radiolabeling. Recombinant plasmids containing HPV type 6C, 11, 16, and 18 DNAs were kindly provided by L. Gissman and E. deVilliers (Deutsches Krebsforschung Zentrum, Heidelberg, Federal Republic of Germany). For use as probes in the in situ filter hybridization and Southern blot hybridization methods, the HPV DNAs were excised from the plasmids to avoid possible false-positive reactions because of the presence of pBR322 sequences in some clinical specimens (2). The HPV DNAs were not excised from plasmids when they were used as probes in the in situ tissue hybridization method to enhance the signal associated with the probe (22). The DNA was labeled with either [^{32}P]dCTP or [^{35}S]dCTP (New England Nuclear Corp., Lachine, Quebec, Canada) with a commercial nick-translation kit (Bethesda Research Laboratories, Gaithersburg, Md.), and the instructions of manufacturer were followed. A specific activity of greater than 10^8 cpm/μg of DNA was achieved for each probe. For all hybridization methods, two probe types were used: one consisted of a mixture of HPV types 16 and 18 DNA, while the second consisted of a mixture of HPV types 6C and 11 DNA.

In situ filter hybridization. In situ filter hybridization was performed by a modification of the methods described by Wagner et al. (26) and Grunstein and Hogness (10). Samples (100 μl) were applied under vacuum to a nitrocellulose filter with a 96-well manifold apparatus (Schleicher & Schuell, Inc., Keene, N.H.). The specificity of the hybridization was controlled by testing serial dilutions of Caski cells, which contain 500 copies of HPV type 16 per cell (27), and similar dilutions of human embryonic fibroblast cells, which do not contain HPV DNA. Cell lysis and DNA denaturation were effected by overlaying the filter on Whatman 3MM filter paper that had been previously soaked in 1 M NaCl–0.5 M NaOH for 5 min, and the procedure was repeated once more. The filters were then neutralized by overlaying them twice for 5 min on Whatman 3MM filter paper that had been previously soaked in 1.5 M NaCl–0.5 M Tris buffer (pH 7.4). The filters were air-dried, placed into a plastic bag, and treated with 2 mg of proteinase K per ml in 10 mM Tris (pH 7.8)–0.5% sodium dodecyl sulfate–0.5 mM EDTA. The filters were washed three times in chloroform (5 min each) and then were given one final wash in 2× SSC. The DNA was fixed on the filter by baking at 80°C for 2 h. HPV DNA sequences were detected by a modification of the method described by Thomas (24). Filters were prehybridized for 2 to 3 h at 42°C in a prehybridization buffer consisting of 50% formamide–5× SSC–50 mM sodium phosphate (pH 6.5)–250 μg of sonicated, denatured salmon sperm DNA per ml–5× Denhardt solution, containing 0.02% each of bovine serum albumin, Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.), and polyvinylpyrrolidone. The hybridization buffer consisted of 4 parts of prehybridization buffer, 1 part of 50% (wt/vol) dextran sulfate, and 5×10^5 cpm of ^{32}P-labeled specific DNA probes per ml. Prior to use, the hybridization cocktail was boiled for 5 min at 100°C and then cooled on ice to achieve denaturation of the DNA. Following the methods described in previously published reports (20, 26), hybridization was carried out under stringent conditions at 42°C (which corresponded to 17°C below the melting point [T_m −17°C]) overnight, and the filters were washed four times under nonstringent conditions (T_m −42°C) at 42°C in 2× SSC containing 0.1% sodium dodecyl sulfate, with each wash lasting 1 h. The filters were enclosed in Saran Wrap and exposed for 1 to 3 days to X-ray film at −70°C by using an intensifying screen (Eastman Kodak Co., Rochester, N.Y.). The filters were subsequently washed under stringent conditions (T_m −17°C) by using nonstringent wash conditions, except that the procedure was carried out at 68°C. A final wash in 0.1× SSC–0.1% sodium dodecyl sulfate was carried out at room temperature for 15 min, and autoradiography was done as described above. All autoradiograms were read independently by three observers, and those specimens that were recorded as positive by two or more observers were scored as positive.

Southern blot analysis. Southern blot analysis was done essentially as described previously (18). Briefly, following phenol-chloroform extraction, the DNA was digested with a restriction enzyme (*Bam*HI) and fractionated by agarose gel electrophoresis. The DNA was then transferred to nitrocellulose filter by the technique described by Southern (23) and prehybridized and hybridized as described above for the in situ filter hybridization method. In keeping with standard practice (4, 7, 9, 18), however, hybridization was carried out in 20% formamide at 42°C under nonstringent conditions (T_m −42°C). The filters were then washed as described above for the in situ filter hybridization method. For the purposes of comparison, the results obtained for the Southern blot method under stringent washing conditions were regarded as being comparable to those obtained for the remaining two assays under stringent hybridization and washing conditions. The autoradiograms were scored by three observers as described above for the in situ filter hybridization method.

In situ tissue hybridization. A modification of the method described by Haase et al. (12) was used for in situ tissue hybridization. Paraffin was removed from the tissue sections with xylene, and the tissue sections were sequentially hydrated in descending grades of alcohol. The DNA was denatured in situ by treating the section in 0.2 N HCl for 10

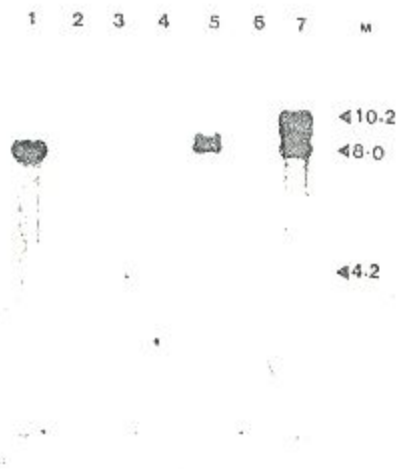

FIG. 1. Autoradiogram of Southern blot hybridization analysis. DNA was extracted from biopsy samples, cleaved with the restriction enzyme *Bam*HI, and analyzed as described elsewhere (18). Lanes 1 to 4, DNA from condylomatous lesions; lanes 5 to 7, DNA from invasive cancer cells. Arrows indicate molecular weight markers (M), in kilobases, as determined by electrophoresis of *Hind*III-digested lambda phage DNA. The blot was probed with HPV type 16 DNA and washed under stringent condition (T_m −17°C). The typical episomal HPV molecule is exemplified by the signal in lane 1, while patterns consistent with viral integration into cellular DNA are exemplified by the signals in lanes 5 and 7.

min, and the sections were then treated with a 25 μg of proteinase K in 0.05 M Tris hydrochloride (pH 7.4) at 37°C. The sections were then treated with a 4% paraformaldehyde solution in phosphate-buffered saline, washed in phosphate-buffered saline containing 0.2% (wt/vol) glycine, followed by a wash in phosphate-buffered saline. The sections were then dehydrated in ascending grades of alcohol and air-dried. Hybridization cocktail contained 2× SSC, 400 μg of denatured salmon sperm DNA per ml, and various concentrations of formamide. Formamide concentrations of 10% (T_m −42°C) and 50% (T_m −17°C) were used for nonstringent and stringent wash conditions, respectively. To the hybridization solution was added 10^5 cpm of ^{35}S-labeled specific HPV DNA per ml. The whole hybridization cocktail was denatured by boiling for 5 min at 100°C, chilled on ice, and made to 10 mM in dithiothreitol (12). Hybridization was carried out at 37°C for 18 h, and the sections were washed as follows: 30 min at room temperature in 2× SSC followed by 1 h at 42°C in 2× SSC. The slides were then washed for 2 to 3 days in three changes of a solution containing 50% formamide, 2× SSC, 0.01 M Tris hydrochloride (pH 7.4), and 0.001 M EDTA. The slides were then rinsed three times for 15 min in 2× SSC and dehydrated in ascending grades of alcohol. Autoradiography was carried out by dipping the slides in melted nuclear track emulsion (NTB2; Kodak Canada Ltd., Toronto, Ontario), which was diluted in distilled water, as directed by the manufacturer. The slides were dried in the dark for 0.5 h and exposed at 4°C for 4 weeks in a light-proof plastic box containing calcium sulfate as a dessicant. Before the slides were developed, they were equilibrated to room temperature and immersed for 3 min in developer (D-19; Kodak), followed by 1 min in 1% acetic acid and 3 min in fixer (Kodak). After 10 min of washing in distilled water, the sections were lightly stained with hematoxylin and then eosin and were independently scored by three readers, as described above for the two other hybridization methods. Cytopreparation of Caski cells and Siha cells, which harbor 500 and 10 copies of HPV-16 (27), respectively, were included to determine the lowest copy number of HPV genomes detectable by this method.

Statistical analysis. The reliability of the hybridization methods in the diagnosis of HPV was examined by percent agreement and by using unweighted Cohen kappa statistics (8). The percent agreement is the number of samples for which identical results were obtained by two methods relative to the total number of samples examined. Use of the kappa statistics is done in an attempt to correct the percent agreement for agreement that occurs by chance alone.

RESULTS

Representative results obtained by each of the three hybridization methods are shown in Fig. 1 to 3. The HPV DNA from condylomatous lesions existed as episomal molecules, as exemplified by the appearance of an 8-kilobase fragment after digestion with the single-cut enzyme *Bam*HI and Southern blot analysis (Fig. 1, lane 1). A similar analysis of HPV DNA extracted from invasive cervical cancer cells sometimes yielded a series of bands (Fig. 1, lanes 5 and 7), a feature which is consistent with the fact that the HPV DNA is integrated into the cellular DNA (4).

The type of hybridization signals observed by the in situ filter hybridization method is shown in Fig. 2. It appears that by this method HPV sequences can be detected within aggregates of cells (26), giving rise to a discrete dot (as exemplified by samples in Fig. 2, rows D and G, column 9, or rows F and G, columns 11 and 12) that fills the whole well. However, no correlation between the number of cells initially present in the samples and the intensity of the signal

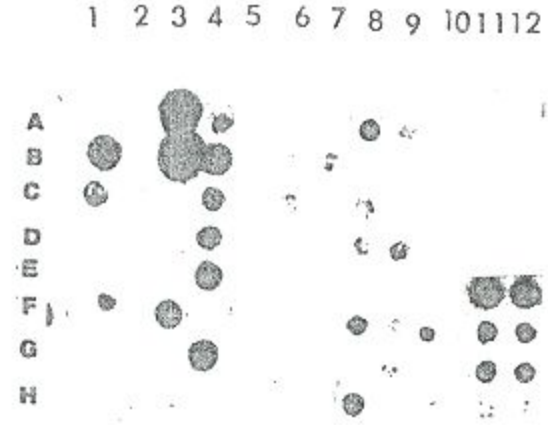

FIG. 2. Autoradiogram obtained by the in situ filter hybridization method. Exfoliated cervical cells were trapped on nitrocellulose paper, lysed, digested with proteinase K, and then hybridized as described in the text. Rows A to H, columns 1 to 9, Clinical samples from patients. The inset (rows A to D, columns 11 and 12) shows that there was no hybridization signal when a 10-fold dilution of human fibroblast cells, ranging from 10^4 to 10^1 cells per well, was included as a negative control. A positive control consisted of similar dilutions of Caski cells, which harbor HPV type 16 sequences (rows E to H, columns 11 and 12). Any samples that showed signals comparable to or weaker than those in row H, columns 11 and 12, were scored negative; and any signal stronger than these were scored positive. A clear positive signal is exemplified by the signal in row A, column 3; and a clear negative signal is illustrated in row A, column 1. Some borderline signals (row A, column 6; row B, column 6; row C, column 2; and row F, columns 6 and 7) are also apparent. These were considered negative for the purposes of the analysis.

was observed. By using various dilutions of HPV type 16 containing Caski cells (Fig. 2, rows E to H, columns 11 and 12), hybridization signals were clearly identifiable in wells that received as few as 100 cells (Fig. 2, row G, columns 11 and 12). Similar experiments with an HPV-negative human fibroblast cell line yielded no detectable hybridization signals at the highest concentration tested (Fig. 2, rows A to D, columns 11 and 12).

When the in situ tissue hybridization method was used for the detection of HPV sequences in condylomatous lesions, the positive silver grains were observed to be unevenly distributed in the different layers of the epithelium. The signals were weakest in the basal layers and progressively increased in intensity toward the terminally differentiated epithelial cells and the koilocytes, which showed the strongest signals (Fig. 3a and b). Positive sections from patients with cancer appeared to show strong reactivity in and around cells, at the periphery of the invasive squamous cell carcinoma islands; but most of the invasive cells were negative (Fig. 3d). Essentially no hybridization signals were seen in the majority of tissues from histologically normal patients, including the morphologically normal epithelium of the columnar cells or the underlying stroma from patients with condyloma and invasive cervical cancer. Occasionally, focal-positive signals were seen in the underlying stroma (Fig. 3f), in areas of inflammatory infiltrate, or along the basement membrane. However, these specimens reacted with all HPV and vector probes under stringent hybridization conditions. Similar observations have been made by Gupta and co-workers (11), and we concur with their conclusions and regard these stromal reactions as nonspecific.

The occurrence of HPV DNAs was found to vary according to clinical condition. Of 26 histologically confirmed condylomas, 23 (89%) were found to contain HPV DNAs by one or more of the methods. A total of 19 specimens (73%) had HPV types 6C- and 11-related DNA sequences; 5 (20%) had HPV types 16 and 18 DNA, and 3 (12%) reacted with HPV types 6C and 11 DNA only under nonstringent hybridization conditions. Among patients with cancer, HPV DNA was found in 19 of 23 (83%) patients by one or more of the methods. These 19 specimens contained HPV types 16- and 18-related DNA, and 13 (57%) also reacted with HPV types 6C and 11 DNA probes. Cervical specimens from women with no histologic evidence of genital neoplasia or condylomas hybridized to HPV sequences to a lesser extent than did those from patients in the other two categories. Among such samples, 4 of 31 (13%) contained such sequences. Interestingly, histologic evidence of metaplasia was found in all the cervices from which positive specimens were obtained.

The occurrence of specific types of HPV according to clinical condition, as determined by each of the hybridization methods, is summarized in Table 1. A total of 62% of the condylomatous lesions were positive for any HPV DNA by the in situ filter hybridization method, 82% were positive for HPV DNA by the Southern blot method, and 72% were positive for HPV DNA by the in situ tissue hybridization method. Among patients with cancer, 89% of the specimens were positive by the in situ filter hybridization method, 70% were positive by the Southern blot hybridization method, and 30% were positive by the in situ tissue hybridization method. Only 9.5% of the specimens from patients with no histologic evidence of neoplasia or condyloma were positive for HPV DNA by the in situ filter hybridization method, while 13% of the specimens were positive by the Southern blot hybridization method and 6% were positive by the in situ tissue hybridization method.

The sensitivities and specificities of each hybridization method were first computed with respect to disease type. The sensitivity of a test is defined as the proportion of subjects with the disease and a positive test result, while the specificity of a test is defined as the proportion of subjects without the disease and a negative test results (8). The results of an analysis in which the patients with condyloma or invasive cancer were pooled are summarized in Table 2. The Southern blot hybridization method exhibited a sensitivity of 76% and a specificity of 87%. The in situ filter hybridization method had a sensitivity of 72% and a specificity of 90%, while the in situ tissue hybridization method had an overall sensitivity of 50% and a specificity of 93%. However, the sensitivity of the in situ tissue hybridization method differed between disease categories: it was 72% for condylomas and 30% for invasive cancers. The relative sensitivities, specificities, and reliabilities of the hybridization methods in detecting HPV sequences are summarized in Table 3. By using the Southern blot hybridization method as the standard, the in situ filter hybridization method had a sensitivity of 66% and a specificity of 88% and showed significant agreement beyond chance (kappa = 0.54; $P < 0.0001$). With respect to the Southern blot method, the in situ tissue hybridization method had a sensitivity of 61% and a specificity of 86% and showed significant agreement (kappa

TABLE 1. Occurrence of HPV type by clinical conditions

Hybridization assay[a]	Histologic diagnosis	No. positive/no. tested (%) for the following HPV types:				
		6 and 11[b]	16 and 18[b]	6, 11, 16, and 18[b]	Other[c]	Total
In situ filter	Condyloma	9/26 (35)	3/26 (12)	1/26 (4)	3/26 (12)	16/26 (62)
	Invasive cancer	4/18 (22)	8/18 (44)	4/18 (22)	0/18 (0)	16/18 (89)
	Normal	2/21 (9.5)	0/21 (0)	0/21 (0)	0/21 (0)	2/21 (9.5)
Southern blot	Condyloma	8/17 (47)	1/17 (6)	2/17 (12)	3/17 (18)	14/17 (82)
	Invasive cancer	0/20 (0)	13/20 (65)	0/20 (0)	1/20 (5)	14/20 (70)
	Normal	1/31 (3)	1/31 (3)	0/31 (0)	2/31 (6)	4/31 (13)
In situ tissue	Condyloma	13/25 (52)	3/25 (12)	2/25 (8)	(0)	18/25 (72)
	Invasive cancer	2/23 (7)	3/23 (13)	3/23 (13)	(0)	7/23 (30)
	Normal	2/33 (6)	0/33 (0)	0/33 (0)	(0)	2/33 (6)

[a] The same number was not available for testing by all three assays.
[b] Specimens were detected under stringent conditions of hybridization for the specific HPV.
[c] Specimens were detected only under nonstringent conditions of hybridization with any of the HPV DNA probes.

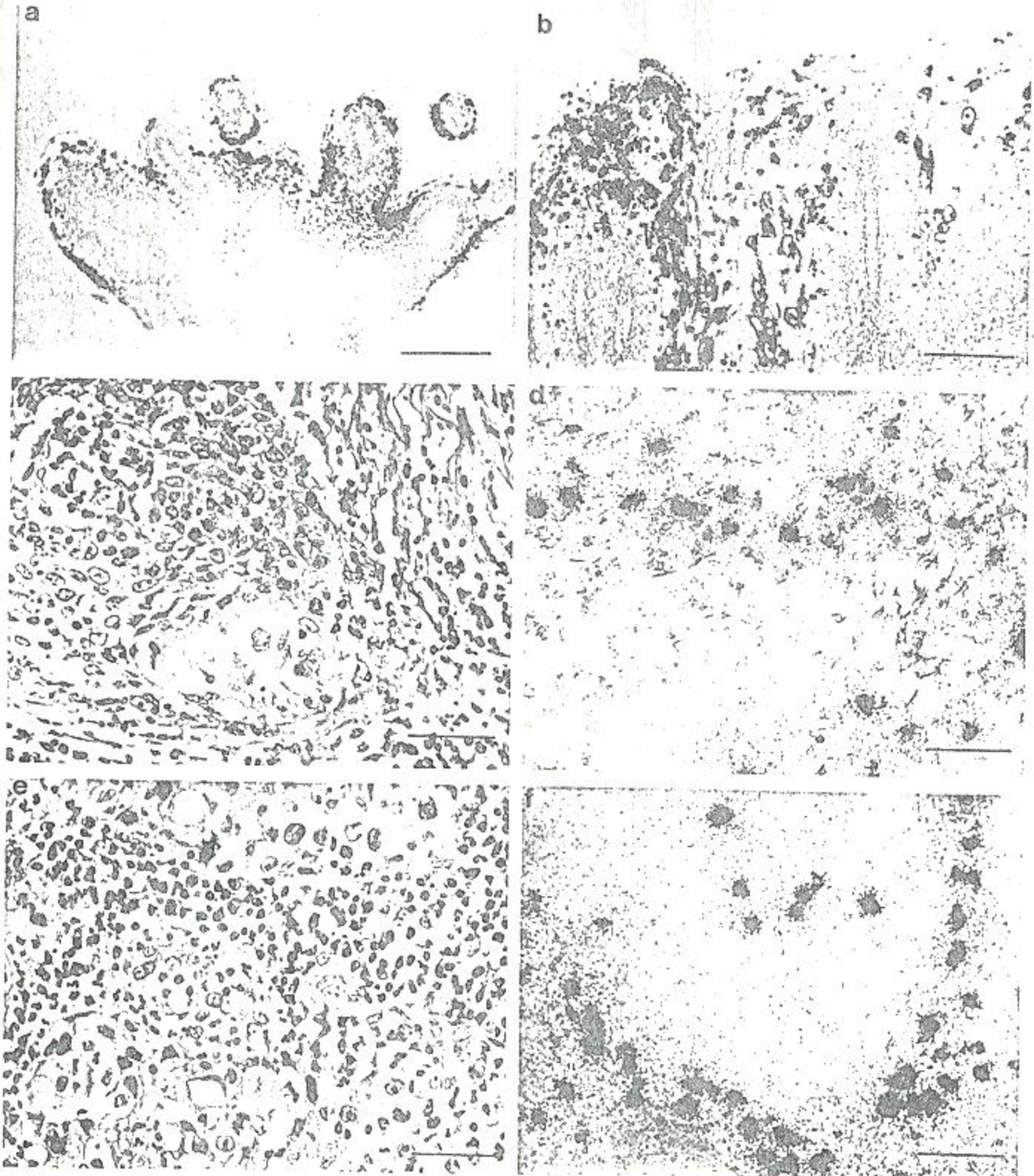

FIG. 3. Autoradiographic signals seen by the in situ tissue hybridization method. Paraffin-embedded biopsy samples were processed by standard histologic methods and hybridized with ^{35}S-labeled HPV probes under stringent hybridization conditions (T_m − 17°C), as described in the text. HPV type 6C and 11 probes were used for condylomatous tissue, and HPV types 16 and 18 probes were used for invasive cancer tissue. (a) Typical condyloma. (b) Higher magnification of positive koilocytes in a condyloma. (c) Hematoxylin staining of an invasive tumor that showed a positive signal in the invasive cells (d). (e) Hematoxylin staining of invasive tumors that showed a positive signal in the stromal cells of the tumor (f). Bar for panel a, 0.2 mm; bars for panels b to f, 0.04 mm.

TABLE 2. Sensitivities and specificities of different hybridization methods in detecting HPV with reference to disease[a]

Hybridization method	No. tested	Sensitivity (%)	Specificity (%)	% Agreement	Cohen kappa statistic	P value
Southern blot	63	76	87	80	0.62	0.0019
In situ filter	65	72	90	78	0.51	<0.0001
In situ tissue	83	50	93	68	0.39	<0.0001

[a] Results for specimens of condyloma and invasive cervical cancer were pooled.

= 0.50; $P < 0.0001$). Pairwise comparison of the in situ filter and in situ tissue hybridization methods similarly showed significant agreement (kappa = 0.55; $P < 0.0001$).

DISCUSSION

Our aim in this study was to compare the sensitivities, specificities, and reliabilities of molecular hybridization methods in detecting HPV sequences under various clinical conditions. To this end, we applied the Southern blot, in situ filter, and in situ tissue hybridization methods to specimens of cervical swabs and biopsies from histologically normal cervices, condylomatous lesions, and invasive cervical cancer cells.

HPV DNA sequences were found in 70% of patients with invasive cancer by using the Southern blot hybridization method, and most of the positive samples contained HPV types 16- and 18-related DNA. This percentage is consistent with that for HPV type 16 reported previously by us (18) and others (7). By the Southern blot hybridization method, HPV DNA was detected in 82% of the condylomatous lesions examined. This percentage agrees with that reported by Crum and colleagues (5), but is somewhat less than that reported by Schneider and colleagues (20). Only 13% of histologically normal ectocervical biopsies were found to contain HPV DNA sequences by the Southern blot hybridization method. This finding is in agreement with the observation of MacNab and co-workers (14) but is lower than that observed by Meanwell and co-workers (15), who found that 35% of histologically normal ectocervices contained HPV type 16 sequences.

Wagner and colleagues (26) adapted the in situ filter hybridization method for detecting HPV DNA in exfoliated cervical cells. Based on the results of the study by Grunstein and Hogness (10), we modified this method to increase the recovery of HPV DNA and digested cellular proteins and mucus to reduce the nonspecific background. Using this modified in situ filter hybridization method, we found that cells from 62% of the condylomatous lesions and 89% of the cervical cancers contained HPV DNA sequences. Our results for invasive cervical cancer cases are in agreement with those of Schneider and colleagues (20), who found HPV DNA in four of four cases. They also detected viral sequences in six of six women with condylomatous lesions, a frequency that was greater than that observed in our study. They also found fewer patients with normal epithelium that was positive for HPV DNA.

Application of the in situ hybridization method to the detection of vegetative replication of PV in tissue sections was first reported by Orth and colleagues (16). Using ^{35}S-labeled probes, we found that 60% of condylomas were positive for HPV types 6C and 11. This rate is in agreement with that observed by Gupta and colleagues (11), who found HPV DNA in 58% of the condylomatous tissues tested, but lower than the 100% positive rate observed by Beckmann and colleagues (3), who used a biotin-labeled probe. Our sample size was significantly larger than those used in previous studies and may represent a more reliable estimate. When we applied the in situ tissue hybridization method to invasive cancer lesions, we found only 30% of the sample to be positive for HPV types 16 and 18 DNA. We also observed that 6% of histologically normal cervices reacted with HPV probes by this method.

The evaluation of the accuracy of a test rests with knowledge of whether the disease is truly present. In our study we evaluated the respective sensitivities and specificities of each of the assays with respect to histologic evidence of disease. Our estimates of sensitivity and specificity could be distorted by selection bias since we defined normal patients as those whose cervices showed no histologic evidence of genital neoplastic or condylomatous disease after hysterectomy. However, about one-third of these cervices were found to have inflammatory or metaplastic changes and were not truly normal. A further source of potential selection bias could result from the fact that we pooled the prevalence data from two different populations to estimate the test parameters. When all the disease categories are considered together, the Southern blot hybridization method gave the highest sensitivity (76%), followed by the in situ filter hybridization method (72%) and finally by the in situ tissue hybridization method (50%). A converse pattern was seen for the specificity; the most specific assay was the in situ tissue hybridization method (93%), followed by the in situ filter hybridization method (90%) and the Southern blot hybridization method (87%). The sensitivity of the in situ tissue hybridization method, however, differed between disease categories, being similar to the other methods in detecting HPV DNA in condylomatous lesions but less sensitive when applied to invasive cancer cells.

The accuracy of one method versus another can also be expressed in terms of relative sensitivity and specificity. When we used the Southern blot hybridization method as a reference to evaluate the other methods for the detection of

TABLE 3. Relative sensitivities and specificities of different hybridization methods in detecting HPV

Hybridization method	No. tested	Relative sensitivity (%)	Relative specificity (%)	% Agreement	Cohen statistic	P value
Southern blot[a] versus in situ filter	56	66	86	80	0.54	<0.0001
Southern blot[a] versus in situ tissue	72	61	86	77	0.50	<0.0001
In situ[a] tissue versus in situ filter	59	66	86	79	0.55	<0.0001

[a] Technique used as a standard in the comparison.

HPV DNA, the relative sensitivities and specificities of the in situ filter and tissue hybridization methods were not different from each other. The percent agreement beyond chance, as computed by the unweighted Cohen kappa statistic, showed good agreement (kappa = 0.50; $P < 0.0001$). It is apparent, however, that agreement was not achieved in a substantial number of cases. This residual lack of agreement could be explained by sampling variation.

Two main types of errors are commonly encountered in assays such as those described here. These include sampling errors, in which material representative of the lesion is not included in the specimen, and interpretive errors in reporting the results of the assay. There is a degree of subjectivity in interpreting the results of the hybridization techniques used in this study, and we attempted to minimize this by accepting the consensus of three independent observers. However, no specific measures were undertaken to control for sampling error. The in situ hybridization method on tissue sections showed that the HPV DNA is predominantly confined to the upper cell layers of the epithelium. For the in situ filter hybridization method, unlike in the biopsy, the surface of the lesion was sampled; thus, an endocervical swab should have captured those positive cells. Use of any sampling method in which a lesion is missed yields a low positive rate, irrespective of the limit of detection of the hybridization method used to test such samples. In this regard, sampling in our study was not colposcopically directed. The in situ hybridization method for tissue section is most vulnerable to this sampling variation, because not only can the lesions be missed by biopsy but they can also be missed by sectioning. Indeed, in some specimens that were tested by the in situ tissue hybridization method, the lesions were poorly represented. Thus, sampling error probably accounted for a substantial amount of the disagreement that was observed.

The relative copy number of the HPV genomes in the various samples may also seriously affect the limit of detection by the hybridization methods. In our hands, a reconstruction experiment indicated that the Southern blot method could detect 0.2 pg of HPV DNA, which corresponds to 0.2 to 0.3 copies of HPV DNA per cell (18). By the in situ filter hybridization method, on the other hand, 0.1 pg of HPV DNA could be detected, an amount that is not identifiable by the Southern blot hybridization method (26). This could account for the slightly higher positivity rate for HPV DNA for cancer cases by the in situ filter hybridization method than by the Southern blot hybridization method. It is difficult to estimate directly the limit of detection of the HPV genomes by in situ hybridization in tissue sections. Even though a single positive cell containing multiple copies of the HPV genome can theoretically be detected by this method, it is possible that fewer than 10 copies of the HPV genome per cell cannot be detected. In support of this argument is our observation that Siha cells, which contain 10 copies of the HPV type 16 genome, as determined by the Southern blot method (27), were negative by the in situ tissue hybridization method (data not shown). With our length of exposure, however, we have been able to detect 500 copies of the HPV genome, as was observed by using Caski cells (27). This limit of detection is slightly better than that observed by Crum and colleagues (5), who detected 800 copies of the HPV genome in their system. Specimens from a number of cancer cases were positive by the Southern blot hybridization method but were negative by the in situ tissue hybridization method. This suggests that the majority of cancers in some lesions might harbor less than 500 copies of the HPV genome per cell.

In conclusion, we found each of the methods to have advantages and disadvantages. While the Southern blot hybridization method permitted the unambiguous identification of the physical state of the HPV genomes and exhibited the highest specificity, it suffered from a reliance on unfixed biopsies and a tedious DNA manipulation that required a special apparatus. The in situ filter hybridization method is a noninvasive technique that is quick and simple and thus well suited for epidemiological studies; however, it suffers from the inability to inherently exclude reactions as nonspecific. The simplicity of the in situ tissue hybridization method and its ability to detect HPV DNA sequences in paraffin-embedded sections lends itself to retrospective studies. Its chief drawback is that it may be of limited sensitivity in detecting HPV sequences in a substantial portion of invasive cervical cancers.

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