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Detection of human papillomavirus in cervical intra-epithelial neoplasia, using in situ hybridization and various polymerase chain reaction techniques

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Abstract One hundred and forty-eight randomly chosen neutral-buffered formaldehyde-fixed cervical biopsies in which cervical intra-epithelial neoplasia (CIN) I–III had been diagnosed were tested for HPV (human papilloma virus) DNA by in situ hybridization (ISH) and polymerase chain reaction (PCR). For ISH, we utilized a biotinylated *pan*probe and type-specific, genomic probe sets. For PCR, we used the general primers GP5/GP6 and their recently described, elongated version GP5+/GP6+, and included the modification of hot-start PCR. Amplified DNA was detected by gel electrophoresis and slot blot hybridization. The positivity rate of ISH was 59% for all biopsies and 69%, 62% and 46% for CIN I, II and III, respectively. The sensitivity of GP5/GP6 was 74% with cold-start PCR and 78% with hot-start PCR. When GP5+/GP6+ was used, the sensitivity increased to 89% with cold-start PCR and to 95% with hot-start PCR. Based on the most sensitive PCR technique, HPV detection was 93%, 95% and 96% in CIN I, II and III, respectively. The number of HPV types decreased with the severity of the lesion, and HPV 16 was the predominant type. Multiple HPVs were rare and almost all HPV-positive cases could be typed. ISH and slot blot hybridization correlated well regarding HPV typing specificity. Our results confirm that distinct HPV types are present in a high proportion of cases of CIN. The sensitivity of ISH is lower than that of PCR. Furthermore, the modified general primers GP5+/GP6+ give a higher yield than GP5/GP6, while hot-start PCR increases sensitivity even further.

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Introduction

Since 1974 a possible role for human papillomavirus (HPV) in the aetiology of human anogenital cancer has been suspected [55], and since 1976 it has been recognized that HPV-induced lesions in the female genital tract are frequently associated with cervical intra-epithelial neoplasia (CIN) and invasive squamous cell carcinoma [23, 24, 47]. Numerous studies have confirmed this correlation [14, 19, 31, 48–55]. At least 27 different HPV types have been associated with the genital tract mucosa. Of these types, HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56 and 58 have been found in cervical carcinomas [10, 39, 40], HPV 16 being the most prevalent type [50].

In situ hybridization (ISH) and polymerase chain reaction (PCR) are molecular techniques that are commonly used for detecting and typing HPV. ISH permits localization of specific nucleic acid sequences at the level of individual cells and can do so with a very high degree of detection specificity [2, 4, 13, 28, 37, 38, 43]. However, its usefulness is occasionally limited by its detection limit of 10–50 copies per cell when biotinylated genomic probes are used [45, 46]. Radioactive labelling has not been found to be more sensitive [4, 37]. Previously, it was shown that HeLa cells (10–50 HPV 18 copies/cell) tested positive with ISH, whereas SiHa cells (1–2 HPV 16 copies/cell) did not [46]. To date, PCR is probably the most sensitive method of HPV testing. Its detection limit has been established as 0.1–1 fg of plasmid HPV DNA when the general primers described below are used [42]. Comparison of the DNA sequences of the genital HPV types 6, 11, 16, 18, 31 and 33 revealed that there were regions of homology within the L1 open reading frame. From these common regions, spanning nucleotides 6764–6883 for HPV 6 and the corresponding regions of the other genital HPVs, the general primers, GP5/GP6,

with template sequences of approximately 140–150 bp, were designed [36]. These primers detect a wide range of HPV types: 6, 11, 13, 16, 18, 30, 31–33, 35, 39, 40, 42–45, 51–59, 61 and 66, and also types that are still unidentified [36, 42]. Recently, they were modified by elongation of their 3′ ends. This modified form, denoted GP5+/GP6+, has resulted in less background noise and therefore provides higher specificity and sensitivity [9].

In this study our aim was to identify different HPV types in CIN by using ISH and PCR on routinely fixed cervical biopsies and to compare the sensitivity and specificity of the two methods. For ISH, we used a biotinylated *pan*probe detecting a wide range of HPVs and type-specific probe sets including the most common HPV types. Various PCR techniques were tested. We compared the general primers GP5/GP6 and their modified version GP5+/GP6+, and also cold-start versus hot-start PCR (where one PCR-dependent reagent is withheld until the temperature reaches 60–80°C to quench mispriming) [11]. The sensitivity of the HPV PCR was determined by a serial dilution of neutral-buffered formaldehyde-fixed SiHa cells. HPV-positive samples were typed using slot blot hybridization and fluorescein labelled, genomic probes.

Materials and methods

Cervical biopsies

The study included 148 women, aged between 18 and 64 (median 33), attending the colposcopy clinic at the Department of Obstetrics and Gynaecology at Uppsala University Hospital; 148 colposcopically directed punch biopsies of the cervix were obtained because abnormal Papanicolaou smears were suggestive of CIN. Fixation of the biopsies was standardized: 4% neutral-buffered formaldehyde solution (4.5 g $NaH_2PO_4{\cdot}H_2O$, 8.3 g $Na_2HPO_4{\cdot}H_2O$, 110 ml concentrated formaldehyde solution and distilled H_2O to give 1 l) (NBF); pH 7.2; and a duration of 15–24 h. The haematoxylin-eosin-stained tissue sections were examined histologically, and the grade of CIN (I, II or III) recorded was based on the proportion of the epithelium occupied by basaloid, undifferentiated cells reflecting the progressive loss of epithelial maturation. Disordered polarity, chromatin clumping and abnormal mitotic figures were further requirements for the diagnosis of CIN. Koilocytosis can be observed in all three stages of CIN, but lesions with only koilocytosis and lacking the classic signs of CIN were not included in the study.

HPV DNA testing and typing by in situ hybridization

The in situ hybridization techniques currently used in our laboratory have been described elsewhere [45, 46]. Briefly, serial 4- 6-µm sections on organosilane-coated slides were baked overnight at 60°C and treated with proteinase K (P-0360, Sigma Biochemicals, St. Louis, Mo.) at a concentration of 0.1 mg/ml 1xSSPE (150 mM NaCl, 10 mM NaH_2PO_4 and 1 mM EDTA) for 30 min at 37°C and 3% H_2O_2 for 10 min at room temperature. Denaturation was done for 5 min on a 94–96°C hotplate. Hybridization was carried out overnight with the *pan*probe (Omniprobe, Digene, Silver Springs, Md.), which detects HPV types 6, 11, 16, 18, 31, 33, 35, 42–45, 51, 52 and 56, and also for 2 h with probe sets 6/11, 16/18 and 31/33/35 (Digene) at 37°C in a moist chamber. Post-hybridization was performed in 30% (Omniprobe) or 50% (probe set) formamide/2xSSPE (final concentration) for 10–20 min at 37°C, followed by enzymatic detection with biotinylated horseradish peroxidase bound to streptavidin (K 377 A-B, Dakopatts, Copenhagen, Denmark) 1:100 for 30 min at 37°C and visualization with 3.3-diaminobenzidine tetrahydrochloride-H_2O_2. Finally, the slides were counterstained in Mayer's haematoxylin for 15–30 s, rinsed in graded ethanols and xylene and coverslipped. A positive (labelled total human DNA to ensure all components were working) and a negative DNA (labelled vector only) hybridization check, provided by the manufacturer, were included to monitor that each ISH step was carried out correctly and that the assay was specific. Further checks were against known HPV-positive biopsies.

DNA preparation and controls

For DNA extraction, one to three 10 µm sections were placed in a 1.5-ml Eppendorf tube containing 150 µl of digestion buffer (50 mM Tris-HCl, pH 8.5, 1 mM EDTA, 0.5% Tween 20) and 0.2 mg/ml proteinase K (1413 783, Boehringer Mannheim, Mannheim, Germany) [21]. Samples were incubated for 4 h at 65°C, and the protease was destroyed by a further incubation for 5 min at 95°C, followed by centrifugation for 5 min in a microfuge at 13,000 rpm. Empty paraffin blocks after each 10th sample and distilled H_2O were used as negative controls. Positive controls were NBF-fixed and paraffin-embedded SiHa cells or patient samples already known to be HPV-positive. To exclude false-negative results, all biopsies were tested with the β-globin primers PC04 5′ CAACTTCATCCACGTTCACC 3′ and GH20 5′ GAAGAGCCAA GGACAGGTAC 3′, resulting in a 268-bp amplimer [30].

Polymerase chain reaction

DNA amplification for HPV was performed on all β-globin-positive biopsies (148/151) with the general primers GP5: 5′ TTTGTT ACTGTGGTAGATAC 3′ and GP6: 5′ GAAAAATAAACTGTAAA TCA 3′ from the L1 region, resulting in a PCR amplimer of approximately 140–150 bp and the recently modified version GP5+: 5′ TTTGTTACTGTGGTAGATACTAC 3′ and GP6+: 5′ GAAAAAT AAACTGTAAATCATATTC 3′. A 50-µl amplification mixture consisted of 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 3.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each primer, 2 U Ampli*Taq* polymerase (Perkin-Elmer, Norwalk, Calif.) and 1–10 µl of each clinical sample. Forty amplification cycles were run on a Perkin-Elmer GeneAmp 2400 according to the following protocol: 1 min denaturation at 94°C, 2 min annealing at 40°C and 90 s extension at 72°C. The initial denaturation was 3 min at 94°C, and the final elongation step was extended to 7 min. Hot-start PCR was performed with AmpliWax (Perkin-Elmer) according to the manufacturer's instructions. The sensitivity of the HPV PCR was checked on a serial dilution of NBF-fixed SiHa cells ranging from 10^3 to 1 cell.

Detection and hybridization

HPV PCR products were initially run on a 1.5% agarose gel and stained with ethidium bromide. Amplified DNA was analysed by slot blot hybridization. The entire genomes of HPV 6, 11, 16, 18, 31, 33, 35, 45, 51, 52, 56 and 58 were labelled with the enhanced chemiluminescent detection (ECL) random prime system (Amersham, London, England) according to the manufacturer's instructions. A portion (10 µl) of the amplimers was bound to a nylon filter by slot blotting using a Minifold II Slot-Blotter (Schleicher & Schuell, Kassel, Germany) and pre-hybridized for 30 min at 60°C in a solution containing 5xSSC (1xSSC contains 150 mM NaCl, 15 mM sodium citrate), 0.5% blocking reagent, 0.1% SDS, 5% w/v dextran sulphate and 100 µg/ml sheared heterologous DNA (e.g. sheared herring sperm DNA, Promega, Madison, Wis.). The labelled probe was denatured for 5 min at 95°C and briefly centrifuged, placed on ice, and subsequently added to the blots. Incubation was continued overnight at 60°C. Post-hybridization washes were carried out in a stringency wash solution of 1xSSC, 0.1% SDS for 15 min at 60°C and in one of 0.5xSSC, 0.1% SDS for 15 min at 60°C. Following the post-hybridization washes, the blots were rinsed in TBS (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) and then incubated in 0.5% blocking reagent in TBS for 60 min at

room temperature. After a brief rinse in TBS, sheep anti-fluorescein-HRP conjugate, diluted 1:1000 in 0.5% BSA/TBS, was applied and the blots incubated for 60 min at room temperature. Signal generation and detection was carried out for 1 min in the solutions supplied. The blots were placed DNA side up in the film cassette and exposed to Cronex 4 film (Du Pont) for 5–15 min.

Results

All negative and positive controls for ISH were satisfactory. The positivity rate was 59% for all biopsies and 69%, 62% and 46% for CIN I, II and III, respectively.

Table 1 HPV-positive cases in CIN stages I–III using ISH. *M* Multiple probe sets, *X* unclassified HPVs, positive with *pan*probe only. Probe set 16/18 was most frequently detected, and the highest proportion of positive cases was found in CIN I

Histopathological diagnosis	HPV type						
	*Pan*probe	6/11	16/18	31/33/35	M	X	*n*
CIN I	31	3	19	6	4	0	31/45
CIN II	34	0	20	11	0	2	34/55
CIN III	22	0	15	4	1	2	22/48
Total	87	3	54	21	5	4	87/148

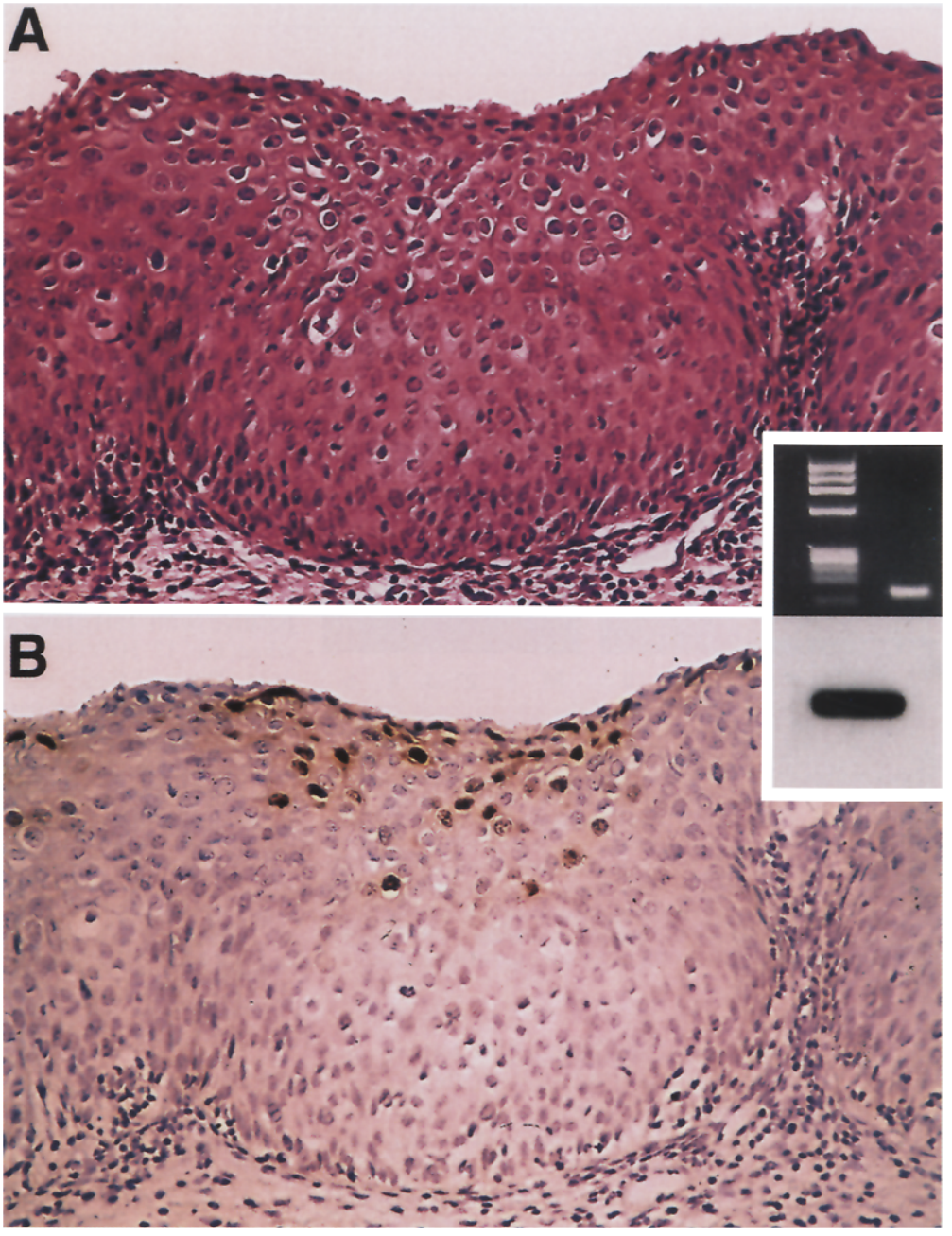

Fig. 1A, B An HPV 16-positive CIN III lesion. **A** H&E, x400. **B** In situ hybridization with probe-set 16/18. Signals are localized to the nuclei of HPV-infected cells in the upper half of the epithelium, x400. The result of the agarose gel electrophoresis including the expected 150-bp polymerase chain reaction (PCR) product and the PCR-slot blot hybridization with the type-specific probe for HPV 16 is shown in the *inset*

Table 2 Presence of HPV DNA in CIN using various PCR techniques. *M* Multiple HPVs, *X* unclassified HPVs. GP5/GP6 and cold start was the least sensitive method, GP5+/GP6+ and hot start the most sensitive one. All cases that were positive with GP5/GP6 were also positive with GP5+/GP6+. The same applied to cold versus hot start PCR. HPV 11 and 51 were not detected

PCR technique	HPV type												
	6	16	18	31	33	35	45	52	56	58	M	X	*n*
GP5/GP6 cold start	3	61	9	9	7	7	2	0	5	2	3	2	110/148
GP5/GP6 hot start	3	65	9	9	8	7	2	0	5	2	3	2	115/148
GP5+/GP6+ cold start	3	69	12	10	10	8	2	1	5	3	3	5	131/148
GP5+/GP6+ hot start	3	70	13	13	11	9	2	1	5	5	3	5	140/148
Total	3	70	13	13	11	9	2	1	5	5	3	5	140/148

Table 3 HPV-positive cases in CIN I–III using GP5+/GP6+ hot start PCR and slot blot hybridization. *M* Multiple HPVs, *X* unclassified HPVs. In CIN I, II and III, 9, 8 and 6 different HPVs were detected respectively, HPV 16 being the most prevalent. The proportional increase of the additionally positive cases as compared with GP5/GP6 cold start PCR was as follows: highest for HPV 58 (150%) and unknown HPVs (150%) followed by HPV 52 (100%); lowest for the otherwise frequent HPVs 16 (15%), 35 (29%), 18 (44%), 31 (44%) and 33 (57%). Of these cases, we found 9 in CIN I (30%), 10 in CIN II (33%) and 11 in CIN III (37%)

Histopathological diagnosis	HPV type												
	6	16	18	31	33	35	45	52	56	58	M	X	*n*
CIN I	3	18	3	5	1	1	0	1	3	3	2	2	42/45
CIN II	0	27	5	5	5	3	2	0	2	1	1	1	52/55
CIN III	0	25	5	3	5	5	0	0	0	1	0	2	46/48
Total	3	70	13	13	11	9	2	1	5	5	3	5	140/148

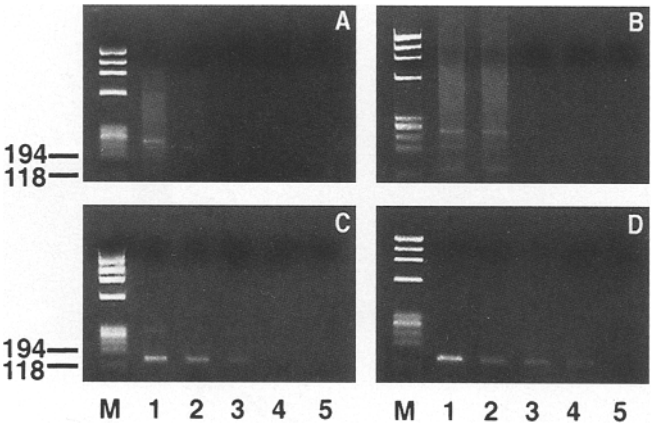

Fig. 2A–D Dilution series of NBF-fixed SiHa cells ranging from 10^3 to 1 cell. One SiHa cell contains 1–2 HPV 16 copies [M molecular marker ΦX 174/*Hae*III; *lanes 1–5* correspond to 10^3, 10^2, 10, 1 SiHa cell and negative control (distilled H_2O)]. **A** GP5/GP6 and cold-start PCR; **B** GP5/GP6 and hot-start PCR; **C** GP5+/GP6+ and cold-start PCR; **D** GP5+/GP6+ and hot-start PCR. Note the background noise using GP5/GP6 compared with GP5+/GP6+. The HPV-specific band is barely visible with GP5/GP6 and cold-start PCR. Sensitivity increased approximately by a factor of 10 for each different application, resulting in a factor of 10^3 between the least and most sensitive PCR technique used in this study

The distribution of different HPVs in CIN I–III is summarized in Table 1, and one HPV-positive CIN III case is illustrated together with its PCR data in Fig. 1A, B.

All negative and positive controls for PCR were satisfactory. The results of the four PCR techniques used are listed in Table 2. The sensitivity of GP5/GP6 was 74% with cold-start PCR and 78% with hot-start PCR. The application of GP5+/GP6+ increased sensitivity to 89% with cold-start PCR and to 95% with hot-start PCR. The distribution of HPVs in CIN I–III is compared in Table 3. HPV detection was 93%, 95% and 96% in CIN I, II and III, respectively. The sensitivity of the dilution series on NBF-fixed SiHa cells is illustrated in Fig. 2A–D.

The remaining results are based on the most sensitive PCR technique: GP5+/GP6+ and hot-start.

Three cases (2%) reacted with more than one HPV type-specific probe, and two of these three were also positive with ISH. Five cases (3%) were not positive when the 12 HPV type-specific probes included were used. Three of these were also positive on ISH with all probe sets in the same part of the epithelium. These signals disappeared after high-stringency washes.

In all women in the entire study population, equal degrees of HPV infection was seen and the variety of HPV types seemed similar. Though most (127, aged between 18 and 44) of the women were infected with HPV 16, the remaining 21 women aged between 45 and 64 had an equal predominance of HPV 16 and 18.

The specificity of PCR-slot blot hybridization and that of ISH correlated well, and these results are compared in Table 4.

Discussion

A significant risk of developing invasive squamous cell carcinoma has been ascribed to infections with HPV [14, 19, 31, 47–53, 55]. Our results confirm that distinct HPV

Table 4 Correlation of PCR-slot blot hybridization and ISH. *M* Multiple HPVs and probe sets, *X* unclassified HPVs. Of all tested cases, one proved HPV DNA positive with ISH but negative with PCR. This CIN III case was positive with the *pan*probe only, explaining the numbers in parentheses. Two cases, positive with *pan*probe only could be typed with PCR-slot blot hybridization. A few cases tested with ISH cross-hybridized to related types not included in the probe sets

ISH	PCR-slot blot hybridization											
	6	16	18	31	33	35	45	56	58	M	X	*n*
*Pan*probe	0	1	0	0	0	1	0	0	0	0	1	3 (4)
6/11	3	0	0	0	0	0	0	0	0	0	0	3
16/18	0	43	7	0	0	0	1	3	0	0	0	54
31/33/35	0	0	0	8	6	4	0	0	3	0	0	21
M	0	0	0	0	0	0	0	0	0	2	3	5
Total	3	44	7	8	6	5	1	3	3	2	4	86 (87)

types are possible mediators in the development of CIN. Only high-risk HPV was found in ≥95% of CIN II and III. CIN I harboured an almost equal amount of HPV (93%), including only a few cases of low-risk HPV. This high frequency is certainly due to our strict requirements for all grades of CIN, as explained in "Materials and methods". Similar figures were recently found in a Japanese and a North American study [22, 31]. Furthermore, the histological review of HPV-negative lesions, initially diagnosed as CIN I, revealed a significant proportion of nondiagnostic cases in a Belgian study [7]. Overall, the prevalence of HPV detection in the current study is consistent with that in other, similar studies. The possible loss of L1 sequences following integration of viral DNA into the host genome has to be considered [41]. In this case a false-negative result of PCR with the GP5+/GP6+ primers would be expected for the eight negative cases. However, a general deletion domain in the integrated viral genome has been located to the E1 open reading frame (ORF) at the 3′-half, and at the 5′-terminus of the E2 ORF [15].

Fewer HPV types were detected in advanced lesions. This finding is consistent with those of other investigations [8, 22]. Like others [8, 18, 50], we found that HPV 16 was the predominant type regardless of the technique being used. Multiple infections were rare, as reported by Cornelissen et al. [6], and almost all cases could be typed. There was no convincing difference in HPV detection with age and CIN. ISH and PCR-slot blot hybridization correlated well on HPV typing. Both methods appear to be specific and reproducible in the detection of HPV. Type-specific probe sets were also reactive with related HPV types not included in the probe sets. Two cases that were positive with the *pan*probe only, but could be typed by slot blot hybridization, showed very weak viral signals in single nuclei. These nuclei were probably lost by consecutive sectioning during testing for the type-specific probe sets.

Not unexpectedly, the sensitivity of PCR was greater than that of ISH, if it is borne in mind that the HPV types detected by PCR-slot blot hybridization were also included in the *pan*probe (except HPV 58, which, however, cross-reacted with probe set 31/33/35). This is in agreement with other studies [3, 7, 34]. All cases but one that were positive with ISH were also positive with PCR. The one case that was showed strong viral signals in a few nuclei with the *pan*probe and could possibly harbour an HPV type not within the detection range of GP5+/GP6+. Although ISH with genomic, biotinylated DNA probes has advantages, including the correlation of morphological diagnosis to molecular biological finding, its present sensitivity is limited to the detection of 10–50 viral copies per cell. The change in detection sensitivity was mostly observed in CIN II and III lesions. Similar detection rates for CIN II and III by means of ISH with biotinylated probes have been published by others [25, 28, 29]. Moreover, it has been reported that the amount of HPV DNA diminishes with increasing severity of the lesion [26, 32, 33]. The ISH assay used here was optimized in earlier studies [45, 46]. Amplification of detection by use of an extra label of antisera after probe hybridization has not improved sensitivity [17] (J. Zehbe). The clinical use of fluorescence label [35] and the recently described biotinyl-tyramide deposition amplification method [20] must await further investigations.

The multiplicity of HPV types requires the use of degenerate, general primers for running PCR. This, in combination with low annealing temperatures, allows the detection of a broad spectrum of HPV types but often results in unwanted and unspecific products owing to mispriming. These products compete with the target fragments for primers and enzyme and may prevent efficient target amplification, especially in rare template reactions. In this investigation, we used GP5/GP6 and their modified, elongated version GP5+/GP6+. The reason for the higher positivity rate with the latter is most probably its higher specificity, which reduces the formation of unwanted products and promotes amplification of the desired sequence. This was further confirmed by the application of hot-start PCR [11], which increased sensitivity even further. With the advent of AmpliWax, hot-start PCR has also become considerably easier to perform, eliminating the risk of sample-to-sample contamination. The detection sensitivity of single viral copies in NBF-fixed SiHa cells was only achieved by using GP5+/GP6+ and hot-start PCR. Without these modifications, sensitiv-

ity decreased by a factor of 10^3 and mispriming was marked, as shown in the dilution series of SiHa cells (similarly also in some clinical samples; data not shown). This, however, is connected with the formaldehyde fixative. In unfixed cell suspensions, amplification is usually more effective [1, 12, 42], though background noise has been reported with GP5/GP6 [42]. It is noteworthy that the highest relative increase in additionally positive cases was seen for HPV 58, unknown HPVs and HPV 52. The lowest in prevalence was HPV 16, followed by HPVs 35, 18, 31 and 33, probably because there are fewer base mismatches in this group.

Routinely fixed biopsies are suitable for testing HPV, provided that NBF is used and fixation time does not exceed 24 h. This finding is consistent with those in other studies [16, 27]. However, it must be stressed that PCR protocols have to be adjusted to detect single HPV copies in a dilution series of SiHa cells (see above). Numerous DNA extraction methods used on archival biopsies have been published [5, 21, 33, 44]. Lungu's quick and easy method gave reproducible results and amplifiable DNA in almost all cases. The paraffin need not be removed for DNA extraction with proteinase K, which is performed at 65°C, allowing the paraffin to melt. After incubation, the samples are centrifuged and protected by a convenient paraffin cover. In this state, they can be kept at 4°C for up to 1 month. For longer storage, we recommend –20°C. Repeated freezing and thawing should be avoided.

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