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Use of Multiple PCR Primer Sets for Optimal Detection of Human Papillomavirus

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Using multiple PCR primer sets, we tried to optimize the detection of human papillomavirus (HPV) in DNA samples isolated from 361 frozen biopsy specimens from patients with invasive cervical carcinomas. The HPVs detected were placed into three distinct groups, including group I/I^{neg} at Telelab (Skien, Norway) and group I^{neg} and group II at the Norwegian Radium Hospital (Oslo, Norway). The consensus primer sets were Oli-1b–oli-2i, My09-My11, Gp5-Gp6, and Gp5+-Gp6+ from the HPV L1 gene and CpI-CpIIG from the E1 gene. Using these consensus primers together with the type-specific primers from E6-E7, we found that 355 patients (98%) were HPV positive. Type-specific primers for HPV types 11, 16, 18, 31, 33, and 35 detected more HPV-infected patients than the most sensitive consensus primer set, while the three consensus primer sets My, Gp/Gp+, and Cp together detected more HPV-positive patients than the type-specific primers. Testing of sensitivity of the PCR with SiHa cells serially diluted in lymphocytes (HPV-negative cells) indicated a detection limit of 6,300 HPV type 16 DNA copies with consensus primers (My, Gp+, and Cp) and 126 original HPV type 16 DNA copies with type-specific primers. Comparison of the amplification results for consensus L1 primers and type-specific E6-E7 primers indicated the presence of L1 deletions in 23 of 56 samples. The conclusion is that in PCR detection systems, multiple consensus primers and type-specific primers should be used in order to detect all patients harboring HPV.

Cervical carcinoma is closely associated with human papillomavirus (HPV). More than 73 HPV genotypes have been isolated to date; types 16, 18, 31, 33, 35, 39, 45, 52, and 58 are considered to belong to the group that puts patients infected with these types at high risk for cervical carcinogenesis (8). Specific viral genes (E6 and E7) from HPV types 16, 18, and 33 act as oncogenes (16, 29). Their expression seems to be necessary, but not sufficient, for conversion to malignancy (42). HPV types 16, 18, and 33 are often found integrated into the chromosomes of cancerous cervical cells, and this integration has been postulated to result in increased levels and stabilities of gene E6 and E7 expression (3, 9, 19). Jeon and coworkers (19) have also demonstrated that HPV type 16 (HPV-16) DNA integration correlates with increased levels of expression of the viral E7 protein and with a selective growth advantage over cells harboring extrachromosomal HPV-16 DNA. Integration often disrupts the integrity and expression of the E1 and E2 open reading frames, which may affect the transcription of E6 and E7 genes (1, 19, 34, 35). In HPV-16 and HPV-18, the E2 proteins are active in virus proliferation, control E6-E7 gene expression, and are necessary for episomal virus production (34). The existence of HPV-negative cervical cancers is now debatable; zur Hausen and de Villiers (42) have argued that if they do exist, their incidence should be low. With recent technology, HPV DNA has been detected in more than 95% of carcinomas of the uterine cervix (2, 5, 20, 23, 27), while other reports indicated lower percentages of HPV in samples from patients with cervical cancer (4, 6, 17, 24, 25, 30, 31). The use of a single PCR primer set for HPV detection is likely to result in an underestimate of the incidence of HPV-positive cancers. This is because the E1 and L1 genes, which are suitable targets for consensus primers, are not necessarily retained in all tumors, while the E6 and E7 genes, which are thought to be retained in all cervical carcinomas, are too variable to be targeted with consensus primers. Thus, targeting E1 or L1 will detect many, possibly all, HPV types, but the target will not always be retained. On the other hand, targeting the E6 and E7 genes will detect all HPV-positive patients, but only those infected with specific HPV types. We report here the results of HPV detection in a large series of patients with cervical carcinoma by using multiple PCR primers from the E1, E6-E7, and L1 regions of the HPV genome.

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MATERIALS AND METHODS

During the period from 1988 to 1993, 361 biopsy specimens were obtained from patients with histologically verified cervical carcinomas (FIGO stages IA to IVB) treated at The Norwegian Radium Hospital. The cervical carcinoma specimens included specimens from 302 squamous cell carcinomas, 32 adenocarcinomas, and 27 adenosquamous carcinomas. Fresh tumor specimens were obtained before therapy, snap frozen in liquid nitrogen, and stored at −70°C.

DNA isolation. The samples were studied in two different laboratories. DNAs were extracted from the first group (group I/I^{neg}) of 220 cervical carcinoma samples at Telelab (Skien, Norway) in 1989, and DNAs were extracted from the other group (group II) of 141 cervical cancer samples at The Norwegian Radium Hospital (Oslo, Norway) in 1993. Four breast carcinoma specimens from group II and lymphocytes from group I/I^{neg} were used as HPV-negative cells in the DNA isolation and PCR amplification procedures.

(i) Group I/I^{neg} specimens. Group I/I^{neg} biopsy specimens were incubated in phosphate-buffered saline containing 2% sodium dodecyl sulfate (SDS) and 500 μg of proteinase K (Boehringer, Mannheim, Germany) per ml for 2 to 3 h or until the biopsy specimen dissolved. The lysate was extracted three times with an

TABLE 1. Primers used in the study

Type	Primer	Primer sequence	Position	Length (bp)
HPV-11	Pr1	5′-CGC AGA GAT ATA TGC ATA TGC-3′	221–240	80
	Pr2	5′-AGT TCT AAG CAA CAG GCA CAC-3′	291–301	
HPV-16	Pr1	5′-TCA AAA GCC ACT GTG TCC TGA-3′	421–440	119
	Pr2	5′-CGT GTT CTT GAT GAT CTG CAA-3′	521–540	
HPV-16[a]	Pr3	5′-GTC AAA AGC CAC TGT GTC CT-3′	420–440	499
	Pr4	5′-CCA TCC ATT ACA TCC CGT AC-3′	899–919	
HPV-18	Pr1	5′-CCG AGC ACG ACA GGA ACG ACT-3′	533–553	172
	Pr2	5′-TCG TTT TCT TCC TCT GAG TCG CTT-3′	682–705	
HPV-31	Pr1	5′-CTA CAG TAA GCA TTG TGC TAT GC-3′	3835–3875	153
	Pr2	5′-ACG TAA TGG AGA GGT TGC AAT AAC CC-3′	3963–3988	
HPV-33	Pr1	5′-AAC GCC ATG AGA GGA CAC AAG-3′	567–587	211
	Pr2	5′-ACA CAT AAA CGA ACT GTG GTG-3′	758–778	
HPV-35	Pr1	5′-CCC GAG GCA ACT GAC CTA TA-3′	610–629	230
	Pr2	5′-GGG GCA CAC TAT TCC AAA TG-3′	821–840	
My[b]	My09	5′-CGT CCM ARR GGA WAC TGA TC-3′	7015–7034	450
	My11	5′-GCM CAG GGW CAT AAY AAT GG-3′	6583–6602	
Gp[b]	Gp5	5′-TTT GTT ACT GTG GTA GAT AC-3′	6624–6646	150
	Gp6	5′-GAA AAA TAA ACT GTA AAT CA-3′	6724–6746	
Gp+[b]	Gp5+	5′-TTT GTT ACT GTG GTA GAT ACT AC-3′	6624–6649	150
	Gp6+	5′-GAA AAA TAA ACT GTA AAT CAT ATT C-3′	6719–6746	
Cp[b]	CPI	5′-TTA TCW TAT GCC CAY TGT ACC AT-3′	1942–1964	188
	CPIIG	5′-ATG TTA ATW SAG CCW CCA AAA TT-3′	1777–1799	
Oli[b]	Oli-1b	5′-TGY AAA TAT CCW GAT TAT WT-3′	6321–6340	323
	Oli-2i	5′-GTA TCI ACI ACA GTA ACA AA-3′	6644–6623	
Oli pr18	Oli-18	5′-GCT TCA CCT GGC AGC TGT GT-3′	6462–6481	158
	Oli2A	5′-GTA TCT ACC ACA GTA ACA AA-3′	6600–6619	

[a] New primers disigned with the Oligo program (National Biosciences).
[b] The position given is on the HPV-16 genome.

equal volume of phenol-chloroform (1:1), ethanol precipitated, and resuspended in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

(ii) Group II specimens. The tissue samples in group II were crushed under aluminum foil with a hammer following freezing with liquid nitrogen. The crushed tissue was then incubated in 1 ml of sterile 1× TNE buffer (0.05 M Tris-HCl [pH 9], 0.15 M NaCl, 5 mM EDTA) with 1% SDS and 500 μg of proteinase K per ml overnight. To avoid cross-contamination the clear solutions were extracted with an equal volume of phenol-chloroform only once; this was followed by an incubation in an equal volume of chloroform. The extracting mixture was rotated carefully for more than 1 h in a rotary mixer, and the upper phase was carefully pipetted off with a Pasteur pipette. After increasing the salt concentration to 0.2 M NaCl, the DNA was precipitated with 2 volumes of 98% ethanol and was left at −20°C for more than 20 min. The precipitated DNA was centrifuged for 20 min at 13,000 rpm and washed once with 70% ethanol. The DNA was precipitated again, dried at room temperature overnight, and dissolved in double-distilled water at room temperature for more than 1 day.

HPV detection. Preparation of PCR reagents, sample preparation, and PCR setup and analysis of PCR products were performed in three separate rooms with dedicated equipment. Filter pipette tips were used for all pre-PCR manipulations. The primers used are listed in Table 1.

Specimens from group I/I^{neg} ($n = 220$) were analyzed by PCR at Telelab with the Oli primers from the gene. Specimens from group II ($n = 141$) were analyzed by PCR with all of the primers except the Oli primers, and specimens from group I^{neg} ($n = 30$) were analyzed with all of the primers.

(i) Group I/I^{neg} specimens. The consensus primer set Oli-1b–Oli-2i (18) was used to amplify a 323-bp segment of the L1 gene. Conditions for amplification were as follows: 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphate, (dNTP) 80 pmol of each primer, and 1.5 U of *Taq* polymerase per 100 μl of the reaction mixture. Samples of 10 μl containing 1 μg of DNA were denatured at 100°C for 7 min before the addition of 90 μl of the PCR buffer mixture. Amplification was performed in a Hybaid TR1 thermal cycler in 500-μl Robbins cap-lock tubes with a 50-μl oil overlay. The PCR program consisted of 95°C for 5 min (95°C for 30 s, 30°C for 30 s, and 65°C for 90 s) for 40 cycles.

The amplified products (10 μl) were applied to 2% agarose gels, and the gels were electrophoresed for 90 min at 130 V. The gels were stained with ethidium bromide, and the bands were visualized by photography on Polaroid MP4 film under UV illumination at 302 nm with a Wratten 23 A filter (Kodak, Rochester, N.Y.).

The HPV type was determined by dot blot hybridization of the amplified samples to ^{32}P-labelled, 20-bp oligonucleotide probes specific for HPV types 16, 18, and 33. Duplicate 10-μl aliquots of each amplified sample were denatured in 100 μl of 0.4 N NaOH–1 mM EDTA and were applied to nylon membranes (Hybond N; Amersham, Buckinghamshire, United Kingdom) or polyvinyl difluoride (Immobilon; Millipore, Bedford, Mass.). Prehybridization (30 min) and hybridization (2 to 16 h) were performed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS–0.25% skimmed milk powder. The filters were washed three times with 6× SSC–0.1% SDS for 20 min. The signals were detected by autoradiography (4 days of exposure).

All samples were analyzed both by gel electrophoresis and by hybridization to each of the three oligonucleotide probes. The results were scored by the following protocol: HPV negative, no signal by hybridization or gel electrophoresis; HPVX (not HPV type 16, 18, or 33), no signal by hybridization and an unambiguous signal (323-bp band) by gel electrophoresis; and HPV types 16, 18, and 33, hybridization signal to the appropriate oligonucleotide probe, with or without a positive signal by gel electrophoresis. HPV-negative samples were reanalyzed with multiple primers (group I^{neg}).

(ii) Group II specimens. Three pairs of consensus primers were used (Table 1). The My primers My09 and My11 of the L1 gene (25) were used with the following final 50 μl of PCR solution: 0.5 to 20.0 μl of the DNA, 50 mM Tris-HCl (pH 8.4), 10 mM KCl, 2.0 mM $MgCl_2$, 1% β-mercaptoethanol, 0.05% bovine

TABLE 2. HPV detection with different primer sets[a]

Sample	No. of samples in which HPV was detected/total no. tested (%)				
	Oli	My	Gp/Gp+	Cp	Ts[b]
Group I/I^{neg}	190/220 (86)	NT[c]	NT	NT	181/220 (82)
Group I^{neg}	0/30 (0)	13/30 (43)	5/30 (17)	9/30 (30)	19/30 (63)
Group II	NT	129/141 (91)	128/141 (91)	125/141 (89)	131/141 (93)

[a] Thirty samples from group I/I^{neg} were tested with all of the primers used to test the samples from group II.
[b] Ts, type-specific probes used in group I/I^{neg} and type-specific primers used in group I^{neg} + group II.
[c] NT, not tested.

serum albumin, 0.2 mM (each) deoxynucleoside triphosphate, 20 pmol of each primer, and 2.5 U of *Taq* polymerase (Boehringer Mannheim). The PCR program consisted of 94°C for 5 min (94°C for 30 s, 45°C for 60 s, and 72°C for 60 s) for 40 cycles and 72°C for 10 min. The Gp primers Gp5 for Gp6 and Gp5+-Gp6+ of the L1 gene (7, 36) were used under the same conditions used for the My primers, but with the following exceptions: 1.5 mM $MgCl_2$ and 50 pmol of each primer were used, and the PCR program consisted of 94°C for 5 min (95°C for 30 s, 44°C for 60 s, and 72°C for 90 s) for 40 cycles and 72°C for 10 min. The Cp primers of the E1 gene (38) were used under the same conditions used for the My primers, but with the following exceptions: 3.0 mM $MgCl_2$ (pH = 8.8), 17 pmol of Cp-I, and 26 pmol of Cp-IIG were used, and the program consisted of 94°C for for 5 min (95°C for 60 s, 55°C for 60 s, and 72°C for 120 s) for 35 cycles and 72°C for 10 min.

Consensus PCR products were detected by hybridization to consensus probes. The Amersham enhanced chemiluminescence kit (RPN3031) was used for labelling, hybridization, and detection of fluorescein-labelled consensus amplimer probes. The My, Cp, and Gp primers were used to amplify HPV type 11, 16, 18, 31, and 33 plasmid DNAs (3, 9, 11), and these PCR products were mixed into two pools containing HPV type 16, 18, 31, and 33 DNAs and HPV type 11, 16, 18, and 33 DNAs before labelling. Eleven-microliter aliquots of the amplified DNA from the patients' samples were run on 7.5% polyacrylamide gels and were transferred to nylon membranes (Hybond N$^+$; Amersham) by using an electroblotting instrument (Bio-Rad, Hercules, Calif.). The DNA was fixed to the membranes by exposure to 302-nm light from a UV transilluminator (Vilber Lourmat, ●●●, France). The membranes were prehybridized for 30 min, and then 45 μl of the fluorescein-labelled probe was added and hybridization was allowed to proceed overnight. After washing and signal generation according to the manufacturer's instructions, the signals were detected by exposing the membranes to Hyperfilm ECL (Amersham) for 2 to 120 min.

HPV type 11, 16 (1/2), 16 (3/4) (new primers), 18, 31, 33, and 35 type-specific primers from the HPV E6-E7 gene region were used as described previously (13), i.e., as described for the My primers but with 1.5 mM $MgCl_2$. The PCR program consisted of 94°C for 5 min (95°C for 30 s, 57°C for 30 s, and 72°C for 60 s) for 35 cycles and 72°C for 10 min. The HPV type-specific PCR products (11 μl) were stained with ethidium bromide (Sigma, St. Louis, Mo.) or SYBR Green I (Molecular Probes, Eugene, Oreg.) in a polyacrylamide gel (7.5%), and samples with positive results were registered directly without subsequent hybridization. Type-specific PCR was performed in sequence. Thus, all patients were tested for HPV types 16, 18, and 33 (a few samples were not tested with the HPV-33 primer set because of a lack of DNA); we tested for HPV types 11, 31, and 35 only in patients who were HPV type 16 and 18 negative.

Analysis was performed in two stages. In the first stage, samples containing 100 to 400 ng of DNA were amplified with the Gp, My, and Cp primers and were hybridized to the appropriate HPV type 16, 18, 31, and 33 probe pool. Samples negative in stage 1 were reamplified by using a fourfold greater amount of input DNA and were hybridized to the HPV type 11, 16, 18, and 33 probe pool. The Gp primers were not used in stage 2; they were found to be less sensitive than the other two primer sets. Samples negative by testing with the Gp primers were instead retested with the modified primers Gp+ (Table 1). The products were detected on SYBR Green I-stained polyacrylamide gels. The low background of the gel obtained with Gp+ primers, in combination with enhanced sensitivity obtained by using SYBR Green I, made hybridization unnecessary in this step.

(iii) Group I^{neg} specimens. DNAs from specimens from 30 HPV-negative patients in group I/I^{neg}, as determined with the Oli primers, were further analyzed by using all of the primers used with the samples from group II (Table 1). We did not perform a hybridization of the consensus primer products with samples from this group because of a reduced background level after increasing the annealing temperature to 47°C for the My primers and 57°C for the Cp primers and because of the increasing fluorescence of the PCR products produced by the new dye used for DNA staining, SYBR Green I (22). Control primers amplifying the conserved regions of the p53 gene (14) and the chromosome 3p (primers from D3S32 [21]) were used to test the quality of DNA from the HPV-negative patients.

Determination of PCR sensitivity by SiHa titration. SiHa cells (one to two copies of HPV-16 DNA per cell) were diluted in extraction buffer (TNE) to give samples containing approximately 420,000, 42,000, 8,400, 4,200, 840, 420, 84, 42, and 8 cells. Each of these dilutions was mixed with about 100,000 HPV-negative immortalized lymphocytes and were digested overnight with proteinase K and phenol extracted as described for group II samples. After precipitation and washing with ethanol, the isolated DNA was dissolved in 200 μl of sterile water. Ten microliters from each of these DNA solutions was tested with primers Gp, My, and Cp and the HPV-16 type specific primer set as described for the group I^{neg} samples.

RESULTS

Of the entire series of 361 specimens from patients with cervical carcinoma, 355 (98%) were HPV positive with at least one of the primer pairs used. All negative PCR controls and negative cell controls which were tested several times with all of the primers used in connection with the different groups were negative. The distribution of HPV-positive samples among the different PCR primer sets used is provided in Table 2. For the 141 samples in Group II, no major difference in sensitivity was observed between primer sets My (91%), Gp-Gp+ (91%), and Cp (89%). Replacement of the Gp primer set with the improved primer set Gp+ combined with SYBR Green I staining increased the sensitivity from 65 to 91%. The use of the Oli primer set in combination with oligonucleotide probes for the 220 samples in group I/I^{neg} gave a lower level of sensitivity (86%). Retesting of 30 Oli primer set-negative samples (group I^{neg}) with primer sets My, Gp+, and Cp and the type-specific primers detected the presence of HPV DNA in 26 (87%) of these samples. Of these, 16 (53%) were detectable with generic primers and 19 (63%) were detectable with type-specific primers. Eighteen samples contained types normally detected in the Oli primer system (HPV types 16, 18, and 33), while five samples contained types not specifically detected in the Oli system (HPV types 11 and 31 and HPVX). This indicates that the lower level of sensitivity found with the Oli

TABLE 3. Single and multiple infections in samples from groups I/I^{neg} and II[a]

HPV type	No. (%) of samples		
	Group I/I^{neg} and group I^{neg}	Group II	Group I/I^{neg}, group I^{neg} and group II
16	145 (66)	92 (65)	237 (66)
18	28 (13)	17 (12)	45 (12)
31	NT[b]	3 (2)	4 (1)
33	16 (7)	9 (6)	25 (7)
35	NT	1 (0.7)	2 (0.5)
11	1 (0.5)	5 (4)	6 (2)
HPVX	10 (5)	11 (7)	21 (6)
16 and 18	5 (2)	0 (0)	5 (1)
16 and 33	4 (2)	0 (0)	4 (1)
16, 18, and 33	1 (0.5)	0 (0)	1 (0.3)

[a] The numbers of samples showing single HPV infections are not counted with samples having multiple HPV types.
[b] NT, not tested.

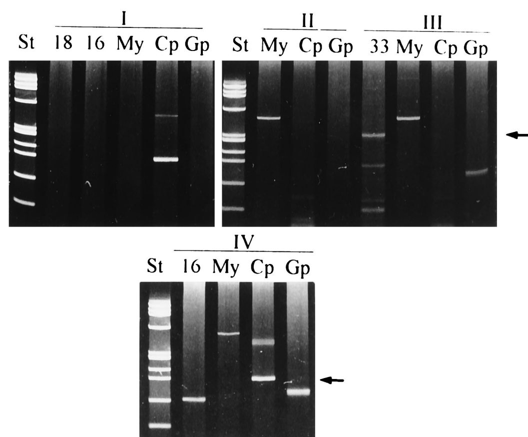

FIG. 1. Consensus PCR products in four typical cervical cancer samples: samples I, II, III, and IV. Lanes 18, 16, and 33, PCR products from type-specific primers detecting HPV 16, 18, and 33, respectively; lanes My, Cp, and Gp, PCR products from consensus primers My09-My11, CpI/CpIIG and Gp5-Gp6, respectively; St, molecular mass standard. Sample I was positive only with the Cp primers. Sample II was positive with the My primers and with the HPV-16 type-specific primers (data not shown). Sample III was positive with the HPV-33, My, and Gp primers (arrows). Sample IV was positive with HPV-16, My, Cp (see arrow) and Gp primers.

primers is due primarily to the less efficient detection of common HPV types (types 16, 18, and 33) rather than failure to detect HPVX.

The generic primers My, Gp/Gp+, and Cp differed markedly in their ability to detect HPV in the group I^{neg} samples, detecting HPV in 13 (43%), 5 (17%), and 9 (30%) of the samples, respectively. The six samples negative with all HPV primers were successfully amplified by using control primers for the human p53 gene or a region of chromosome 3. These samples therefore contained amplifiable DNA and can be regarded as HPV negative.

Multiple infections were not observed in samples from group II, but 10 (5%) multiple infections were observed among the samples in group I/I^{neg} and group I^{neg} (Table 3). Six of these multiple infections were found only with the Oli primers and oligonucleotide probes, while the rest were found among the Oli primer-negative samples. Three multiply infected samples negative with the Oli probes were also negative with one or three of the other consensus primers. The loss of one or two different types of Gp, Cp, and My PCR products in four typical carcinoma samples is illustrated in Fig. 1 and the results are summarized in Fig. 2. The loss of only one of the consensus PCR products was observed for 56 samples (16%), while the loss of Gp/Gp+ and My consensus PCR products together was observed for only 9 samples (Fig. 2). By testing with one of the type-specific primers, 23 of the 56 samples also showed PCR products from the E6 gene. PCR of DNAs isolated from serially diluted SiHa cells mixed with HPV-negative lymphocytes showed an average detection limit of 6,300 HPV-16 DNA copies (1 to 2 copies in each cell) for the consensus primers and an average of 126 HPV-16 DNA copies for the type-specific primers (Table 4).

DISCUSSION

In order to evaluate the role of HPV in initiating cervical carcinoma, it is necessary to use optimal techniques for HPV detection and typing. The risks from using PCR as a detection instrument are both overdiagnosis because of contamination and underdiagnosis because of suboptimal primer selection and other technical problems.

One contamination risk before DNA isolation is the crushing of tissue with a mortar. There is always a risk of transferring virus particles when using this type of equipment. The group I/I^{neg} samples were therefore transferred directly into a tube with buffer and proteinase K before homogenization with a Pasteur pipette. The group II samples were frozen in liquid nitrogen and were crushed under aluminum foil (new foil was used for each sample) with a hammer before it was transferred to the proteinase K buffer. As an extra precaution, we also included DNA isolation controls with both water and HPV-negative cells. The precipitated DNA, which was dissolved in double-distilled water, had to be properly dissolved (more than 2 days in a rotator) before use in the PCR. We found that undissolved DNA may produce false-negative HPV PCR results.

Some recent studies obtained high HPV incidence rates similar to those obtained in our study, whereas other studies reported lower incidence rates (Table 5). In the present study, HPV DNA was identified in 98% of the invasive cervical carcinomas, and only six samples were negative with all the primer sets. Since we did not find any difference in the HPV detection rate between adenocarcinoma and squamous cell carcinoma samples (20) we did not include evaluation of tumor types in the present study.

In 635 cervical cancer samples from Spain, Colombia, and Brazil, HPV was detected in only 85% of samples by using only one consensus primer set (10, 30). Guerrero et al. (12) found an even lower rate of HPV positivity (69%) by using similar methods. This may be due to differences in the true rate of HPV positivity, but it may also be due to differences in the choice of primers and staining methods. We used four consensus primers and six type-specific primers and a new DNA stain, SYBR Green I, which can increase the visible fluorescence from PCR products in polyacrylamide gels 100 times compared with ethidium bromide (22). We and others (25) have demonstrated that DNA isolated from 10^7 SiHa cells in different

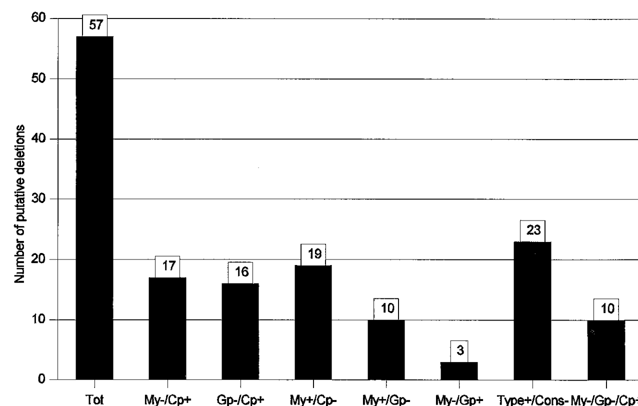

FIG. 2. Loss of consensus PCR products. Tot, all the samples in group I^{neg} and group II that lost one of the consensus PCR products; My−/Cp+, samples negative with the My primer set but positive with the Cp primer set; Gp−/Cp+, samples negative with the Gp primer set but positive with the Cp primer set; My+/Cp−, samples negative with the Cp primer set but positive with the My primer set; My+/Gp−, samples positive with the My primer set but negative with a combination of the Gp and Gp+ primer sets; My−/Gp+, samples negative with the My primer set but positive with a combination of the Gp and Gp+ primer sets; Type+/Cons−, samples negative with one of the consensus primer sets but positive with the type-specific primer set; My−/Gp−/Cp+, samples negative with both the My and the Gp primer set but positive with the Cp primer set.

TABLE 4. Titration of SiHa cells mixed with 100,000 immortalized lymphocytes at each dilution[a]

Primer set	Result for following no. of cells:									
	4	8	42	84	420	840	4,200	8,400	42,000	420,000
Gp	−	−	−	−	−	−	+	+	+	+
Cp	−	−	−	−	−	−	+	+	+	+
My	−	−	−	−	−	−	+	+	+	+
16 pr1/2	−	−	−	+	+	+	+	+	+	+
16 pr3/4	−	−	−	+	+	+	+	+	+	+

[a] PCR results with Gp, Cp, and My consensus primer sets and 16 Pr1-Pr2 and 16 Pr3-Pr4 type-specific HPV16 primer sets.

dilutions can permit the detection of HPV-16 DNA corresponding to 10 to 100 SiHa cells. However, this situation is not comparable to that in the environment in patient tissues, in which HPV is not present in all the epithelial cells. However, if the SiHa cells, alike in our study, are mixed with normal cells and diluted before DNA isolation, the lower limit of PCR detection with consensus HPV primers is 4,200 SiHa cells (6,300 HPV-16 DNA copies). Earlier values of PCR sensitivity for a few copies of HPV DNA in clinical samples may be misleading, since the determinations were not made in a DNA-rich, natural setting, and the titration measurements and estimations were based on purified DNA and not on cells. The high level of sensitivity of PCR with the type-specific primers may explain why some of the samples positive by PCR with the type-specific primers were negative by PCR with consensus primers. Template deletion may also explain the loss of PCR product in carcinomas.

The variation in the HPV detection rate from 54 to 93% by different investigators (Table 5) using the My primers may be related to difficulties in using primers that amplify long PCR products in paraffin-embedded tissues or in samples with degraded DNA. Different DNA isolation methods, the accuracy of the sample size and tissue quality, the amount of DNA in the PCR solution, and PCR conditions may also explain the variations. There was a higher level of variation in the sensitivities of the primers with group I^{neg} samples than with group II samples. This is probably due to the fact that the DNA samples from group I/I^{neg} samples had been isolated from the cancer tissue 6 years earlier and the quality may have deteriorated.

No background PCR product bands were observed when detecting HPV in clinical samples with the new Gp5+–Gp6+ primers, even with variations in the PCR conditions. These new primers, which amplify a short PCR product and which have the highest degree of sensitivity in cervical cancer samples, may therefore be the most useful primers for the detection of HPV. The Cp primers had lower levels of sensitivity than the Gp+ primers, and they produced more extra bands in PCRs with cancer samples than the Gp+ primers. However, the Cp primers may be more sensitive when they are used for HPV detection directly, and the product obtained with Cp primers stained with SYBR Green I without hybridization (the Cp probe mixes are not consensus probe mixes).

In accordance with Van den Brule and colleagues (39), we found differences in HPV positivity between the consensus primers, showing that there may be deletions or mutations of the L1 or E1 templates (Fig. 1). Monk and colleagues (28) found 11 samples that were positive with type-specific primers or probes from the E6 gene but that did not produce any signals with the consensus L1 primers. Munoz et al. (30) and Guerrero et al. (12) also found samples positive by Southern blotting and ViraPap (9 and 12 samples, respectively) which were negative with the My (L1) primers. Wagatsuma et al. (41) and Matsukura et al. (26) also found deletions in the L1 and E1 genes from cell lines. Loss of the consensus PCR product in 18 of our 56 group I^{neg} and group II samples (Fig. 2) may be due to variations in the primer affinity, since the PCR-positive primers gave low optical densities in the gels. The rest of the samples (38 samples) from which consensus PCR products were lost should have deletions or mutations in either E1 or L1 since they gave clear and strong signals with one or two of the consensus primer sets. Among the cervical samples in our study, three samples tested with the Gp/Gp+ primers from L1 were strongly PCR positive, while they were negative with the My primer set from L1, and 9 samples were PCR positive with the My primer set but negative with the Gp/Gp^{+} primers. The Gp/Gp+ primers were considered to be positive for samples negative with the My primer set, indicating a possible deletion or mutation in L1 between the annealing area of the My and the Gp/Gp+ primers. All except 2 of the 10 samples negative with the Gp/Gp+ primer sets were only weakly PCR positive with the My primers. For the two samples for which the PCR products of the My primers were considered to be clearly positive, changes among nucleotides in the Gp/Gp+ primer annealing area may have occurred. Consequently, HPV infections in patients with cervical carcinoma may remain undetected if only one consensus primer set is used for HPV detection. Therefore, a combination of consensus primer must be included in a future PCR system to find all the HPV-positive cases of cancer. Hybridization of the PCR products did not increase the sensitivity of our method but added to the specificity.

Ten of the samples in group I/I^{neg} and group I^{neg} had multiple infections, while group II did not contain any samples with multiple infections. Four of the samples infected with HPV types 16 and 33 cases may have been infected with HPV type 16 or 33 variants since these types belong to the same HPV group (group A [32]).

Several samples positive for HPV but whose PCR products produced faint signals upon staining might have had a low viral load. A low viral load may, however, also indicate a more truncated and integrated HPV genome. Strong signals in the gels by PCR for most of our cervical carcinoma samples may indicate a high viral load, even though our method is not quantitative. The importance of the viral load in carcinomas is as yet unknown and should be studied further in relation to tumor progression.

TABLE 5. HPV detection of cervical carcinomas by different investigators

Reference	No. of cervical carcinoma samples analyzed	% Detection with the following primer set:			
		My	Gp	Cp	Type 16
Eluf-Net. et al. (10)	186		84		54
Van den Brule et al. (39)	21		91		84
Monk et al. (28)	218	79			44
Herrington et al. (15)	114	54			
Prussia et al. (33)	20			90	65
Ter Maulen et al. (37)	53		89		38
Guerrero et al. (12)	302	69			48
Williamson et al. (40)	68	81			46
Meijer et al. (27)	112		100		
Karlsen et al. (20)	143	91	9[a]	89	63

[a] Combination of the old Gp primer set and the new Gp+ primer set.

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