

# Group-Specific Differentiation between High- and Low-Risk Human Papillomavirus Genotypes by General Primer-Mediated PCR and Two Cocktails of Oligonucleotide Probes

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**In recent years, general primer-mediated PCR assays have been developed to detect a broad spectrum of human papillomavirus (HPV) genotypes. In this study, a procedure enabling a simple group-specific differentiation of high-risk (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -54, -56, and -58) and low-risk (HPV-6, -11, -34, -40, -42, -43, and 44) HPVs following an HPV general primer-mediated (GP5+/GP6+) PCR is presented. By computer-assisted sequence analysis, oligonucleotides (30-mers) specific for 19 different HPV genotypes were selected from the internal part of the 150-bp GP5+/GP6+-amplified region. These oligo probes were tested for specificity in a Southern blot analysis of PCR products derived from the same panel of HPV types. No cross-hybridizations were found. The sensitivities of the oligo probes varied from the femtogram level for the well-amplified HPV types like HPV-16 and -18 to the picogram level for the less-well-amplified HPV types like HPV-39 and -51. These sensitivities were reached when the oligo probes were applied both individually and in a cocktail. On the basis of these results, two cocktail oligo probes that enabled a specific and sensitive differentiation between low- and high-risk HPV types were composed.**

At present, more than 70 human papillomavirus (HPV) types have been isolated, of which more than 30 infect the cervical mucosa (7, 8). PCR-based HPV detection assays are increasingly used to determine HPV prevalence in cervical biopsies and scrapes. To ensure the detection of a broad spectrum of HPV genotypes, general or consensus primer-based PCR assays have been developed (10, 11, 13, 21, 27, 30, 33, 35, 41). Together with simple and rapid pretreatment methods for cervical scrapes, these assays have made it possible for HPV-positive and HPV-negative scrapes to be distinguished. However, subsequent HPV typing was often incomplete and limited to more common HPV types, in particular HPV-6, -11, -16, -18, -31, and -33 (39). Thus far, HPV typing has been performed either by sequencing (29), restriction enzyme analysis (20), and HPV type-specific hybridization of PCR products (21) or by additional type-specific PCR for a number of HPV types (37). By now it is known that at least 15 HPV types (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -54, -56, -58, -59, and -66 and ME180) are associated with cervical carcinomas and carcinomas in situ and are considered high-risk HPV types (1, 5, 8, 19, 32). A different subset of HPV types (HPV-6, -11, -34, -40, -42, -43, and 44) associated with benign cervical lesions belongs to the group of low-risk HPVs. In addition, on the basis of follow-up studies of women with cervical intraepithelial neoplasia lesions, it appeared that the presence of high-risk HPV types has a predictive value for progressive cervical intraepithelial neoplasia disease (12, 16, 26, 31). Therefore, classification of HPVs into groups with different biological behaviors instead of on an individual basis would be more useful. Consequently, a typing procedure which discriminates among groups of HPV types having different oncogenic potentials has to be devel-

oped. For this purpose, cocktails of type-specific oligonucleotides were used for the hybridization of HPV general primer-mediated PCR (GP-PCR) products to distinguish between high-risk and low-risk HPV genotypes. Oligonucleotide sequences were selected, and the specificity and sensitivity of the oligo probes were determined experimentally on the PCR products of 19 different HPV genotypes generated by the GP5+/GP6+ PCR method (6).

## MATERIALS AND METHODS

**Clones and clinical specimens.** Plasmid clones or cervical scrapes were used to select an HPV test panel containing the following HPV types: 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 54, 56, and 58. HPV clones of types 6b, 11, 16, and 18 were kindly provided by H. zur Hausen and L. Gissmann (Heidelberg, Germany), HPV-40 was provided by E.-M. de Villiers (Heidelberg, Germany), HPV-31 was provided by A. Lorincz (Gaithersburg, Md.), and HPV-33, -39, -42, and -54 were provided by G. Orth (Paris, France). HPV-45 was provided by K. V. Shah (Baltimore, Md.), and HPV-51 was provided by G. Nuovo (New York, N.Y.). Cloned HPV types 35 and 56 were obtained from the American Type Culture Collection (Rockville, Md.). Cervical scrapes containing HPV-44 were obtained from the OLVG Hospital in Amsterdam and had previously been typed by type-specific PCR or successive hybridization with subgenomic HPV probes (5).

**HPV detection.** Isolated DNA from cloned HPVs or 10  $\mu$ l of crude cell suspensions of cervical scrapes was subjected to GP5+/GP6+ PCR as described previously (6). Briefly, standard PCRs were carried out in 50  $\mu$ l containing 50 mM KCl, 10 mM Tris HCl (pH 8.3), 200  $\mu$ M each deoxynucleoside triphosphate, 3.5 mM MgCl<sub>2</sub>, 1 U of thermostable DNA polymerase (Amplitaq; Perkin-Elmer), and 50 pmol each of the GP5+ (5'-TTTGTTACTGTGGTAGATAC TAC-3') and GP6+ (3'-CTATACTAAATGTCAAATAAAAAG-5') primers. A 4-min denaturation step at 94°C was followed by 40 cycles of amplification with a PCR processor (PE9600; Perkin-Elmer). Each cycle included a denaturation step at 94°C for 1 min, a primer annealing step at 40°C for 2 min, and a chain elongation step at 72°C for 1.5 min. The final elongation step was prolonged by 4 min to ensure a complete extension of the amplified DNA. For the specificity analyses, five PCRs were performed for each target DNA to obtain ultimately a pool of 250  $\mu$ l of PCR product for further analysis. Aliquots (10  $\mu$ l) of each PCR product were layered on 1.5% agarose gels and transferred onto positively charged nylon membranes (Qiabran; Westburg) by diffusion blotting in 0.5 N NaOH-0.6 M NaCl.

**Synthesis and labeling of probes.** Type-specific oligonucleotides (30-mers) were selected on the basis of sequence information derived from the EMBL

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TABLE 1. Sequences of HPV type-specific oligonucleotides

HPV probe	Sequence (5' to 3')
Probe 6.....	ATCCGTAAC TACATCTTCCACATACACCAA
Probe 11.....	ATCTGTGTCTAAATCTGCTACATACACTAA
Probe 16.....	GTCATTATGTGCTGCCATATCTACTTCAGA
Probe 18.....	TGCTTCTACACAGTCTCCTGTACCTGGGCA
Probe 31.....	TGTTTGTGCTGCAATTGCAAACAGTGATAC
Probe 33.....	TTTATGCACACAAGTAACTAGTGACAGTAC
Probe 34.....	TACACAATCCACAAGTACAATGCACCATA
Probe 35.....	GTCTGTGTGTTCTGTGTCTTCTAGTGA
Probe 39.....	TCTACCTCTATAGAGTCTTCCATACCTTCT
Probe 40.....	GCTGCCACACAGTCCCCACACCAACCCCA
Probe 42.....	CTGCAACATCTGGTGATACATATACAGCTG
Probe 43.....	TCTACTGACCCTACTGTGCCAGTACATAT
Probe 44.....	GCCACTACACAGTCCCCTCCGTCTACATAT
Probe 45.....	ACACAAAATCCTGTGCCAAGTACATATGAC
Probe 51.....	AGCACTGCCACTGCTGCGGTTTCCCCAACA
Probe 52.....	TGCTGAGGTTAAAAAGGAAAGCACATATAA
Probe 54.....	TACAGCATCCACGCAGGATAGCTTTAATAA
Probe 56.....	GTACTGCTACAGAACAGTAAAGTAAATATG
Probe 58.....	ATTATGCACTGAAGTAACTAAGGAAGGTAC

database or kindly provided by H. Delius (Deutsches Krebsforschungszentrum, Heidelberg, Germany), after alignment analysis with the CLUSTAL program (PC/Gene, release 6.7; IntelliGenetics, Inc.) (14). The oligonucleotides were synthesized by Pharmacia by the methoxy-phosphoramidite method. Twenty picomoles of each of the type-specific oligonucleotides was end labeled with [ $\gamma$ - $^{32}$ P]dATP by means of T4 polynucleotide kinase (Promega) and used individually or in a cocktail. The high-risk oligonucleotide cocktail probe consisted of the oligoprobes for HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -54, -56 and -58. The low-risk oligo probe contained the type-specific oligonucleotides for HPV-6, -11, -34, -40, -42, -43, and -44. The GP-PCR probe (34) consisted of a mixture of GP-PCR products generated from cloned HPV-6, -11, -16, -18, -31, and 33. A total of 2.4 ng of this cocktail was used to generate an [ $\alpha$ - $^{32}$ P]dCTP random primer-labeled probe.

**Southern blot analysis of HPV-specific PCR products.** Membranes were preincubated for 2 h at 55°C in hybridization solution (0.5 M sodium phosphate [pH 7.4], 7% sodium dodecyl sulfate [SDS], 1 mM EDTA). Afterwards, [ $\gamma$ - $^{32}$ P]dATP-labeled type-specific oligonucleotides or the [ $\alpha$ - $^{32}$ P]dCTP-labeled cocktail probe was added, and the hybridization was carried out at the same temperature overnight. Subsequent washings were done three times, with each wash in 3× SSC-0.5% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 30 min at 55°C. Autoradiography was performed for 18 h at -70°C with Kodak Royal X-Omat film and intensifying screens.

TABLE 2. Homology analysis of the GP5+/GP6+ internal oligo probes from 19 genital HPVs

Probe	HPV type(s) (no. of mismatches) <sup>a</sup>
HPV-6.....	11 (7); 34 (10)
HPV-11.....	6 (7); 55 (10)
HPV-16.....	6 (9)
HPV-18.....	45 (10)
HPV-33.....	58 (7)
HPV-35.....	31 (10); 32 (10)
HPV-40 <sup>b</sup> .....	7 (4)
HPV-42.....	32 (10)
HPV-43.....	45 (7); 68 (10); 35 (10)
HPV-44.....	13 (10); 55 (9)
HPV-45.....	43 (7); 7 (10)
HPV-52.....	32 (10); 35 (10)
HPV-56.....	33 (7); 52 (9)
HPV-58.....	33 (7); 52 (9)

<sup>a</sup> Only HPV types showing between 5 and 10 mismatches are indicated. Oligos from HPV-31, HPV-34, HPV-39, HPV-51, and HPV-54 contained more than 10 mismatches with other HPVs.

<sup>b</sup> The only probe for which there were fewer than seven mismatches.

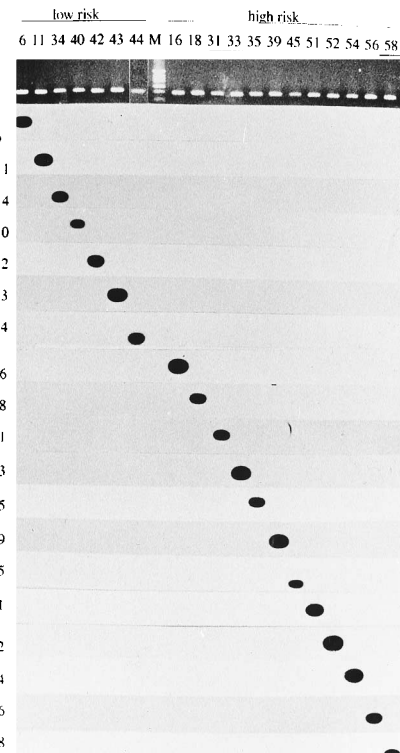


FIG. 1. Specificity analysis of HPV type-specific oligo probes on GP5+/GP6+ PCR-derived amplification products of 19 HPV types. GP-PCR products are shown after electrophoresis on a 1.5% agarose gel stained with ethidium bromide (top gel) and after Southern blot analysis by hybridization with type-specific oligonucleotides (lower gels). Lane M: pBR322 DNA digested with *Hinf*I. The numbers along the top indicate the HPV type, and those on the left side indicate the HPV type-specific probe.

## RESULTS

**Selection of type-specific HPV oligo probes within the GP5/GP6 region.** Regions of heterogeneity (36) within the HPV L1 regions flanked by the GP5+/GP6+ primer pair were used for the selection of oligonucleotides (30-mers) specific for HPV-6, -11, -16, -18, -31, -33, -34, -35, -39, -40, -42, -43, -44, -45, -51, -52, -54, -56, and -58 (Table 1). To determine the specificities of these oligonucleotide sequences, they were aligned to 64 HPV-specific nucleotide sequences containing the GP5/GP6 region. This group of sequences consisted of 61 subgenomic and genomic sequences of cloned HPVs and sequences of three GP-PCR products of still unidentified HPVs (36 and unpublished data). Except for the HPV-40-specific oligo probe, all the oligonucleotides selected contained more than six mismatches with the corresponding regions of the other HPVs. The HPV-40-specific oligonucleotide showed four mismatches with the related HPV-7. The results are summarized in Table 2.

**Specificity of the HPV type-specific oligo probes.** The specificities of the oligonucleotide probes were experimentally determined with the individual radioactively labeled oligonucleotides by Southern blot analysis of the GP5+/GP6+ PCR products were obtained from 1 ng (each) of cloned HPV-6, -11, -16, -18, -31, -33, -34, -35, -39, -40, -42, -43, -44, -45, -51, -52, -54, -56, and -58. As can be seen from Fig. 1, under the conditions of the analyses, all the oligo probes appeared specific for their corresponding HPV genotypes and no cross-hybridization with other HPV types was observed.

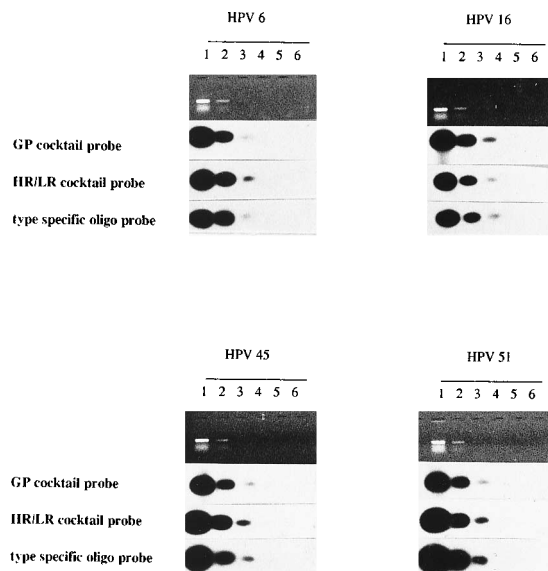


FIG. 2. Sensitivity analysis of HPV probes on GP-PCR products of HPV-6, -16, -45, and 51. GP-PCR products are shown after electrophoresis on 1.5% agarose gels (top gel for each HPV type) and after Southern blot analysis (lower gels). Hybridization was performed with the random-primed-labeled GP-PCR cocktail probe, the high-risk (HR) HPV oligo cocktail probe (panels HPV 16, 45, and 51) or the low-risk (LR) HPV oligo cocktail probe (panel HPV 6), and the corresponding HPV type-specific oligo probe. The concentrations of the GP-PCR products for the different HPVs were as follows: lane 1, 10 ng; lane 2, 1 ng; lane 3, 100 pg; lane 4, 10 pg; lane 5, 1 pg; and lane 6, 100 fg.

**Sensitivity of oligo probe hybridization.** Recently, the sensitivity of the GP5+/GP6+ PCR method appeared to be between 70 copies of viral genome per 20,000 cells for well-matched HPVs (e.g., HPV-16 and -18) and 700,000 copies per 20,000 cells for less-well-matched HPVs (e.g., HPV-39 and -51) after hybridization of the PCR products with the GP-PCR probe (6). To determine the sensitivities of the oligo probes, Southern blot analysis of 10-fold dilutions of the corresponding HPV GP5+/GP6+ PCR products ranging from 10 ng to 100 fg was performed and the signals were compared with those obtained for the random primer-labeled GP-PCR probe originally used (36). For this analysis, HPV types which match well (HPV-6, -16, and -45) or less well (HPV-51) with the GP5+/GP6+ primers were chosen (6). It appeared that the sensitivities of the individual oligo probes (Fig. 2) were similar to the sensitivities obtained with the random primer-labeled GP-PCR probe. The sensitivity obtained with both the GP-PCR probe and oligo probe reached the level of 100 pg for the GP-PCR product (Fig. 2).

#### Analyses of HPV high- and low-risk oligo cocktail probes.

After the establishment of the specificities and sensitivities of the individual HPV type-specific oligoprobes, the high-risk and low-risk oligonucleotide cocktail probes were also analyzed with the panel of HPV GP5+/GP6+ PCR products. In contrast to the original GP-PCR probe, which detects all the genital HPV types analyzed (Fig. 3), the high-risk and low-risk oligo probes clearly differentiated the high- and low-risk HPV genotypes, respectively (Fig. 3). No cross-hybridization of low- and high-risk HPV types was detected with both cocktails.

The sensitivities of the oligo cocktail probes (Fig. 2) appeared to be similar to the sensitivities obtained with the random primer-labeled GP-PCR probe (Fig. 2) and the individual oligonucleotides (Fig. 2).

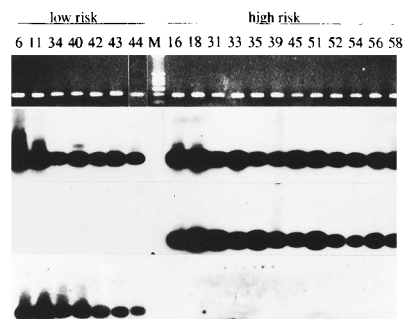


FIG. 3. Southern blot analysis of GP-PCR products after hybridization with the random-primed-labeled GP-PCR cocktail probe (upper middle gel), the high-risk (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -54, -56, and -58) oligo cocktail probe (lower middle gel), and the low-risk (HPV-6, -11, -34, -40, -42, -43, and -44) oligo cocktail probe (lower gel). The top gel represents the amplified products after gel electrophoresis and ethidium bromide staining. Lane M: pBR322 DNA digested with *HinfI*.

## DISCUSSION

During the last few years, increasing evidence that certain HPV types are playing an important role in the development of cervical cancer has been obtained from in vitro transformation studies (18, 22, 24, 42) and epidemiological studies (9, 17, 19, 23, 25, 28). Recently, a phylogenetic tree based on sequence homology was presented, in which the different HPV types could be classified in groups of high-risk (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -58, and -66) and low-risk (HPV-6, -11, -42, -43, and -44) HPV genotypes. This tree indicates that the relatedness of HPV types at the sequence level correlates with their biological behavior (19, 38). This relatedness was recently further substantiated by a follow-up study showing that the continuous presence of related high-risk HPV types was strongly correlated with progressive cervical intraepithelial neoplasia disease (26). Although further research on the correlation between the presence of different HPV types and progressive cervical intraepithelial neoplasia disease is necessary, classification of HPV in groups on the basis of different biological behaviors instead of individual HPV typing is less confusing and will be appreciated by the clinician. In our laboratory, much effort was made to improve the detection of HPV by PCR in a routine setting (40). So that those women having a higher risk for developing cervical cancer could be identified in large screening programs, an assay enabling rapid differentiation of high-risk and low-risk HPVs was developed in this study. It was shown that oligo cocktail probes can be used to specifically differentiate between general primer (GP5+/GP6+)-mediated HPV PCR products derived from selected groups of high-risk and low-risk HPVs. A recent study already showed that despite the strong conservation of the GP5/GP6 region, typing of GP-PCR products was possible by hybridization using subgenomic and genomic probes of 27 mucosotropic HPVs (5). However, utilization of a mixture of these probes resulted in a reduced specificity (data not shown), most likely because strongly conserved sequences, including the primer regions, were present in the probes. The increase in HPV sequence information during the last few years (4) allowed the selection of the oligonucleotide sequences of 22 genital HPVs. These HPVs include the 19 HPVs used in this study (Table 1) as well as HPV-59 and -66 and ME180, which are presently under investigation. For a substantial number of HPV genotypes like those of HPV-6, -11, -33, -43, -45, -56, and -58, no 30-mer oligonucleotide sequences having more than seven mismatches with those of other HPV types (Table 2)

could be selected within the GP5+/GP6+ region. However, the hybridization data (Fig. 1) revealed that the presence of seven mismatches between certain oligonucleotides and related types did not result in cross-reactivity, indicating that all, except one, of the oligonucleotides selected are highly specific. Only the HPV-40 oligo probe revealed fewer than seven mismatches with a related type (HPV-7). It is unknown whether this oligo probe would give rise to cross-reactivity with HPV-7 DNA. However, thus far, HPV-7 has never been detected in the genital mucosa, making it unlikely that cross-hybridization of the HPV-40 oligo probe and HPV-7 would lead to incorrect HPV-40 prevalence data for cervical scrapes. In addition, the amount (1 ng) of pHPV target used in these GP-PCR assays accounts for more than 70 million viral copies. Since this excess of target DNA did not result in cross-reactivity, it is assumed that even high yields of HPV DNA in clinical specimens will not result in cross-reactivity. Since in our experience (5, 6, 9, 12, 23, 26, 34, 37) the amount of HPV DNA rarely reaches the 1-ng level, cross-reactivity in cervical smears can be excluded as a possibility.

The sensitivity data revealed that the efficiency of the hybridization of the oligo probes was 100 pg of the GP-PCR products for all HPV types investigated (Fig. 2). This is similar to the sensitivity obtained with the previously described random primer-labeled GP-PCR probe consisting of a mixture of GP5/GP6 PCR products of HPV types 6, 11, 16, 18, 31, and 33 (6). Consequently, it can be concluded that depending on the number of mismatches between a primer and an input target DNA, between 1 fg and 10 pg of HPV DNA can be detected with oligo probes after GP5+/GP6+ PCR. This corresponds with 0.0035 and 35 HPV copies per cell, respectively (30). Even for the latter case, this sensitivity level is in the range of the level determined with the recently described hybrid capture system for the detection of HPV in cervical scrapes (15). There have been questions concerning the sensitivity of the method for detecting double infections. The method also allows for the discrimination of multiple HPV types in one sample without a loss of sensitivity, even when the types are not present in equal amounts (unpublished data).

In addition, it was shown that the oligo probes exhibit a strong specificity. This means that the oligo cocktail probes are likely to be unable to detect HPV types which are not present in the cocktail. Consequently, these probes can not be used to replace the original GP-PCR probe, which was able to detect a broader spectrum of HPV types, including putative novel high-risk HPV types (36). This would only be possible when all HPV types which are present in all cervical carcinomas worldwide have finally been identified. Therefore, the proposed screening strategy is to perform one general primer PCR assay combined with three hybridization steps of the GP-PCR products. The original hybridization protocol should be maintained for discrimination between HPV-positive and -negative samples (40) and should be followed by oligo cocktail hybridization for the discrimination of the two HPV risk groups. At this moment, the application of the low-risk HPV oligo cocktail probe has been included in the strategy since infection with low-risk HPV types may point to a sexual behavior which makes low-risk-HPV-type-infected women more prone to subsequent infection with high-risk HPV types. Sexual behavior (e.g., age at first intercourse and number of sexual partners) has been recognized as an important risk factor for cervical cancer (2). However, when sufficient follow-up data for women with cytologically normal and abnormal cervical smears containing HPV have been collected, the strategy might be adapted to hybridization with only the high-risk HPV oligo cocktail probe.

The method for the detection of HPV PCR products can

easily be converted to a nonradioactive hybridization format by using DIG-labeled oligo probes after Southern or dot blotting. However, an approach that is even more user friendly would be preferred for robust routine mass screening. An automated enzyme-linked immunosorbent assay reading system for the detection of HPV PCR products would be ideal. This could be achieved by generating PCR products with a biotinylated primer, capturing the products on streptavidin-coated microwell plates, and hybridizing the products with a DIG-labeled oligo (cocktail) probe in combination with immunohistochemical staining of the ultimate hybrid. This system is presently under investigation.

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