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A General Primer GP5+/GP6+-Mediated PCR-Enzyme Immunoassay Method for Rapid Detection of 14 High-Risk and 6 Low-Risk Human Papillomavirus Genotypes in Cervical Scrapings

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Two cocktails of digoxigenin-labeled human papillomavirus (HPV) type-specific oligonucleotide probes and an enzyme immunoassay (EIA) were used as a basis to develop a group-specific detection method for 14 high-risk (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) and 6 low-risk (types 6, 11, 40, 42, 43, and 44) HPVs, following a general primer GP5$^+$/bioGP6$^+$-mediated PCR. The sensitivity of this high-risk/low-risk (HR/LR) HPV PCR-EIA ranged from 10 to 200 HPV copies, depending on the HPV type. Comparison of HR/LR HPV PCR-EIA with radioactive Southern blot hybridization using a general probe on the same PCR products derived from 417 cytomorphologically abnormal cervical scrapings resulted in an overall agreement of 96% between the two methods. Complete concordance between group-specific HR/LR HPV detection and individual typing results for both single and multiple infections indicate the strong specificity of this HR/LR HPV PCR-EIA. Multiple infections could be predicted by comparing PCR-EIA optical density values of the cocktail probes with one of the individual oligonucleotide probes. This novel HR/LR HPV PCR-EIA allows accurate and rapid identification of high-risk and low-risk HPV types in cervical scrapings and will facilitate HPV detection in HPV mass-screening programs.

Epidemiological and molecular biological data indicate that infection with certain human papillomavirus (HPV) types is the main factor involved in the development of cervical cancer (12, 30). About 30 different HPV types have been found to infect the genital mucosa (9, 10). Based on phylogenetic relationship and their presence in benign or malignant cervical lesions, these HPVs are divided into low-risk (LR) and high-risk (HR) HPV types. In this context HPV-6, -11, -40, -42, -43, and -44 are considered LR while HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68 belong to the HR group of HPVs (2, 16, 26).

Several studies have shown the potential relevance of HPV testing in cervical cancer screening programs and management of patients with slight abnormal cytology (4, 5, 15, 19, 22, 29). However, large trials are needed to assess the impact of HPV testing on invasive cancer rates and cost-benefit issues. In order to make HPV testing in cervical scrapings applicable for robust mass screening, the HPV test has to detect at least all HR HPV types in a simple manner.

General or consensus primer-mediated PCR (GP-PCR) assays have been developed to detect a broad spectrum of genital HPV genotypes in one PCR (24, 25). Recently, we have introduced a GP5+/GP6+ PCR-based procedure which included Southern blot hybridization of PCR products with cocktails of radioactively labeled HPV type-specific internal oligonucleotides. In this way, a single assay allowed differentiation between a number of HR and LR HPV types in cervical scrapings (7, 13). Still, this method would be facilitated by converting the radioactive detection procedure into a nonradioactive format.

Recent data indicate that the application of an enzyme immunoassay (EIA) to detect PCR products can be an appropriate nonradioactive alternative (1, 3, 14, 17, 20). Therefore, we aimed in this study to develop an HR/LR HPV PCR-EIA for the detection of 14 HR and 6 LR HPV genotypes as mentioned above. In this method the GP5+/GP6+ PCR was performed as described by Jacobs et al. (13) with the exception that the GP6+ primer was biotinylated (bioGP6+). For EIA analysis, GP5+/bioGP6+-generated PCR products were captured on streptavidin-coated microwells, denatured by alkaline treatment, hybridized to cocktails of digoxigenin (DIG)-labeled internal oligonucleotide probes, and detected immunohistochemically by reading optical density (OD) values at different time intervals (i.e., 1 h, 3 h, and overnight) as previously described (14). For the distinction between positive and negative samples, the OD values obtained after overnight substrate incubation were taken into account. In addition to the HPV type-specific oligonucleotides described before (13), oligonucleotides specific for HPV-59 (TCTACTACTGCTTCTATTC CTAATGTATAC), HPV-66 (TATTAATGCAGCTAAAAG CACATTAACTAA), and HPV-68 (TCTACTACTACTGAA TCAGCTGTACCAAAT) were used in this study.

Several variables, for example, the amount of PCR products to be used for capturing, the probe concentration, the composition of hybridization buffer, and hybridization conditions, have been optimized previously (14). However, the most important problem which we encountered during the development of the HR/LR HPV test was the standardization of the DIG-labeled oligonucleotides to enable equal detection among the different HPV types. Therefore, commercially synthesized DIG-labeled oligonucleotide probes (Eurogentec, Liege, Belgium; Isogen, Leiden, The Netherlands) were ordered. To ensure a high quality of probes, 95% labeling efficiency of oligonucleotides and purification by reverse-phase chromatography were requested.

The analytical sensitivity of the HPV PCR-EIA using these

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oligonucleotide probes in two cocktails was determined on $GP5^+$/$bioGP6^+$ PCR products from 10-fold dilutions of cloned HPV-6, -11, -16, -18, -31, -33, -35, -39, -40, -42, -43, -44, -45, -51, -52, -56, -58, -59, -66, and -68 DNA in a background of 100 ng of human placental DNA. Using a cutoff value of three times the mean OD value of the PCR-negative controls, the analytical sensitivity varied from 0.5 to 10 fg, for both an HR (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68) and an LR (HPV-6, -11, -40, -42, -43, and -44) HPV cocktail probe depending on the genotype. This corresponds to an equivalent of approximately 10 to 200 copies of the viral genome. In Fig. 1 the analytical sensitivity of the PCR-EIA for the HR HPV types 16, 18, 51, and 58 is shown. As described previously (7), these types display a total of 2 (HPV-16 and -18), 5 (HPV-58), and 10 (HPV-51) mismatches with both GP-PCR primers. A similar sensitivity was reached when using the oligonucleotide probes for these HPV types individually (Fig. 1). This sensitivity appeared superior to that obtained with the radioactive procedure described originally (7) in which the sensitivity range was much broader and varied from 1 fg to the picogram level for HPV types which match well and poorly with the GP primers, respectively (7).

To investigate whether this HR/LR HPV PCR-EIA can be performed accurately on clinical specimens, cervical scrapings ($n = 417$) were obtained from the outpatient clinics of the University Hospital Vrije Universiteit, Amsterdam, The Netherlands. Two cervical scrapings were taken to perform both cytomorphological analysis and HPV detection, as earlier described (28). The collected scrapings were classified according to the modified KOPAC classification system as used in The Netherlands (27) and included 328, 64, 17, and 8 cases classified as Pap 3a, Pap 3b, Pap 4, and Pap 5, respectively. This group of cytomorphologically abnormal cervical scrapings was considered ideal for the evaluation of the HR/LR HPV PCR-EIA since a high HPV prevalence and a variety of HPV genotypes could be expected (6).

Cervical scrapings were first prescreened by a β-globin PCR by using the primer combination PCO_3 and PCO_5 as described by de Roda Husman et al. (8) to check the quality of the target DNA. Only cervical scrapings showing successful amplification of β-globin sequences were subjected to GP5+/bioGP6+ PCR.

$GP5^+$/$bioGP6^+$ PCR products derived from the 417 β-globin PCR-positive cervical scrapings were analyzed by both HR/LR cocktail in the EIA and conventional Southern blot hybridization with a radioactively labeled general probe, developed to distinguish between HPV-positive and -negative samples (25). As shown in Table 1, HPV DNA could be detected in 320 specimens after Southern blot analysis. Of these, 304 were positive in the PCR-EIA giving an overall agreement of 96% between the two methods. The EIA-positive cases comprised 273 cases which were positive with only the HR cocktail, 11 cases positive with only the LR cocktail, and 20 cases reacting with both HR and LR cocktails. Of the 113 PCR-EIA-negative samples, 97 scrapings were also negative with the general probe, indicating that 16 HPV-positive cervical scrapings were not identified by group-specific EIA. We anticipate that this might be due to the limited composition of the LR cocktail which represents only 6 different HPV types. Since the unidentified HPVs were mainly found in smears classified as cytomorphologically Pap 3a ($n = 11$) and Pap 3b ($n = 5$) and not in Pap 4 and Pap 5 scrapings, it is indeed likely that these belong to the LR HPV group. This was confirmed by additional oligonucleotide hybridization experiments which showed that HPV types like 26 and 54, originally isolated from nonmalignant epithelium (9, 10), were present in this unidentified group (data not shown).

The high specificity of selected oligonucleotide probes and HR/LR cocktails has previously been demonstrated by radioactive Southern blot analysis in reconstruction experiments (13). However, to determine the specificity on clinical samples, all HR/LR HPV PCR-EIA-positive samples were again subjected to GP5+/bioGP6+ PCR and subsequently typed with the individual oligonucleotide probes by using PCR-EIA and Southern blot analysis. The specificity of HR/LR HPV PCR-EIA can be considered very high based on a complete concordance in HR versus LR HPV detection between the respective cocktail probes and individual oligonucleotide probes and the equal typing results between EIA and Southern blot hybridization. In addition, individual HPV typing revealed 229 samples showing single HPV infections, of which 219 samples contained an HR HPV type and 10 samples contained an LR HPV type. It concerned 18 different HPV types, i.e., 4 LR (HPV-6 [$n = 1$], -11 [$n = 1$], -42 [$n = 5$], and -43 [$n = 3$]) and 14 HR (HPV-16 [$n = 108$], -18 [$n = 17$], -31 [$n = 16$], -33 [$n = 13$], -35 [$n = 8$], -39 [$n = 3$], -45 [$n = 8$], -51 [$n = 11$], -52 [$n = 6$], -56 [$n = 7$], -58 [$n = 13$], -59 [$n = 2$], -66 [$n = 5$], and -68 [$n = 2$]) HPVs. Moreover, a total of 75 specimens contained multiple HPV types comprising 20 different HPV types, i.e., 6 LR HPVs (types 6, 11, 40, 42, 43, and 44) and 14 HR HPVs (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). Although double infections ($n = 54$) prevailed, triple ($n = 17$), quadruple ($n = 3$) and quintuple ($n = 1$) infections were also found. Combinations of HR HPV types ($n = 54$) were most frequently found, followed by HR/LR ($n = 20$) and LR/LR ($n = 1$) combinations.

As shown earlier for HPV-16 and -18 (14), a linear-logarithm relationship between OD values and HPV copy number was also found for the other HR and LR HPV types investigated in this study, provided that the OD values fall within the linear range ($OD \leq 2$). This advantage of the method was further used in the evaluation of multiple HPV infections. Indeed, a correlation was observed between the OD value of the cocktail probe and the cumulative OD values of the oligonucleotide probes. The differences in OD value read between the cocktails and a given oligonucleotide probe after 3 h of substrate incubation were calculated and plotted in a histogram (Fig. 2). It appeared that in the overwhelming majority of samples (80%) an OD difference of >0.2 was attributed to multiple HPV infections. In 33 out of 304 cases OD values were >2 after 3 h of incubation with substrate. These included both single ($n = 23$) and multiple ($n = 10$) HPV infections. For these samples, the data of a 1-h substrate incubation were used for comparison. This means that the criterion of OD values (cocktail versus oligonucleotide probe) between single and multiple infections can be used for the identification of multiple HPV infections.

In this study we have used three times the mean OD value of the PCR negative controls as a cutoff point. Although there is no international standardization for determining this value for HPV PCR-EIA (1, 3, 17, 20), this cutoff value revealed a 100% agreement between EIA results and results from the radioactive method. However, to get more insight in determining a cutoff point, we are in the process to analyze a series of HPV-negative scrapings with the PCR-EIA.

Several EIA-based methods have been described to facilitate HPV DNA detection and typing after general primer-mediated PCR (1, 3, 14, 17, 20). All these methods are restricted to identify a limited number of HPV types. In contrast to the above-mentioned methods, the PCR-EIA described in this study has the advantage of being able to detect 14 unequiv-

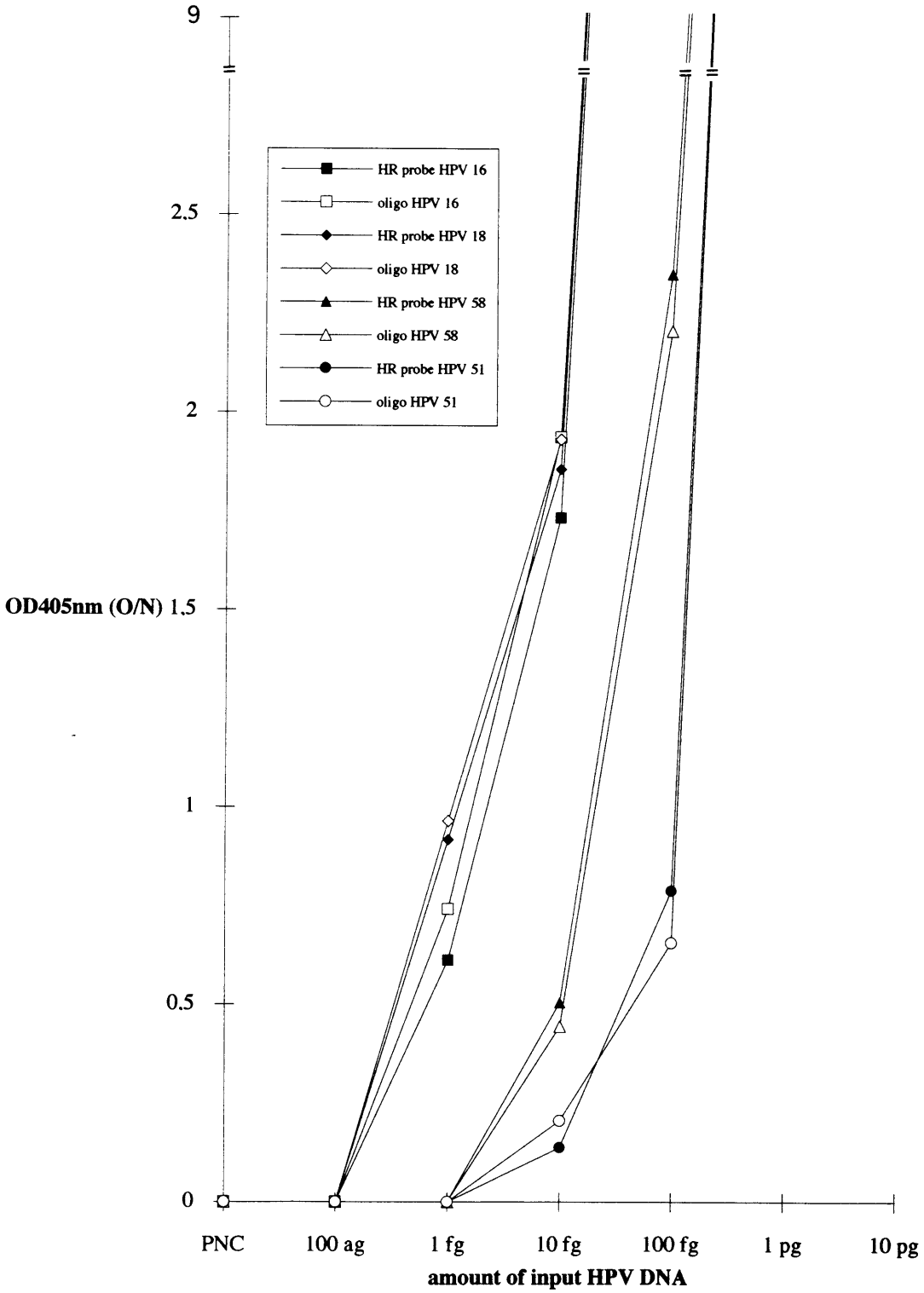

FIG. 1. GP5+/bioGP6+ PCR-EIA sensitivity analysis of cocktails and oligonucleotide probes for the detection of HR HPV types 16, 18, 51, and 58 by using cocktail probes and oligonucleotide probes. ODs obtained after overnight substrate incubation are plotted against the amount of input HPV DNA.

ocal HR and 6 LR types both in groups and individually in one PCR. With this GP5+/bioGP6+ PCR-EIA it is also possible to genotype PCR products generated by the consensus primers MY09/11B (18), since the GP5+/bioGP6+-generated fragments fall within the MY09/11B region. This makes the developed HPV group-specific EIA system universal for the two primer sets most extensively used in HPV studies.

Furthermore, PCR-EIA can be used semi-quantitatively for all HR HPV types (14). This means that the suggested strong predictive value of high viral load for severe cervical lesions in

TABLE 1. Comparison of EIA with radioactive Southern blot hybridization of PCR products for the detection of HPV in cervical scrapings

Cytology	No. of β-globin PCR-positive samples	No. of cervical smears							
		Southern blot analysis of PCR products with a radioactive general HPV probe[a]		EIA					With discrepant results (%)
		HPV negative	HPV positive[b]	HPV negative	HPV positive[b]			Cumulative HPV positive	
					HR probe[c]	LR probe[d]	HR and LR probe		
Pap 3a	328	88	240	99	199	10	20	229	11 (3)
Pap 3b	64	9	55	14	49	1	0	50	5 (1)
Pap 4	17	0	17	0	17	0	0	17	0 (0)
Pap 5	8	0	8	0	8	0	0	8	0 (0)
Total (%)	417 (100)	97 (23)	320 (77)	113 (27)	273 (66)	11 (2)	20 (5)	304 (73)	16 (4)

[a] The radioactive general HPV probe detects HPV types for which specific oligonucleotides are present in the HR and LR probe and additional types (25).
[b] HPV-positive scrapings include single and multiple HPV infections.
[c] HR cocktail probe includes oligonucleotides for HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68.
[d] LR cocktail probe includes oligonucleotides for HPV types 6, 11, 40, 42, 43, and 44.

women with mild dyskaryosis (5) can be further defined and extended for all HR HPV types. Also, the hypothesis that high viral load in cervical scrapings is a marker for HPV persistence and the development of chronic dysplasia (11) can now be further analyzed. We are in the process to apply this semiquantitative PCR in our follow-up studies of women with mild dysplasia (21) and normal cytology (23). Finally, it will be clear that with this method no special measures have to be taken to handle nonradioactive material. Moreover, DIG-labeled oligonucleotide probes can be stored for months without a decrease of quality, which is an important prerequisite for robust HPV testing. Further automation will speed up the whole HPV testing procedure and reduce manual handlings, making large trials to evaluate the value of PCR-based HPV testing in cervical cancer screening programs feasible in the very near future.

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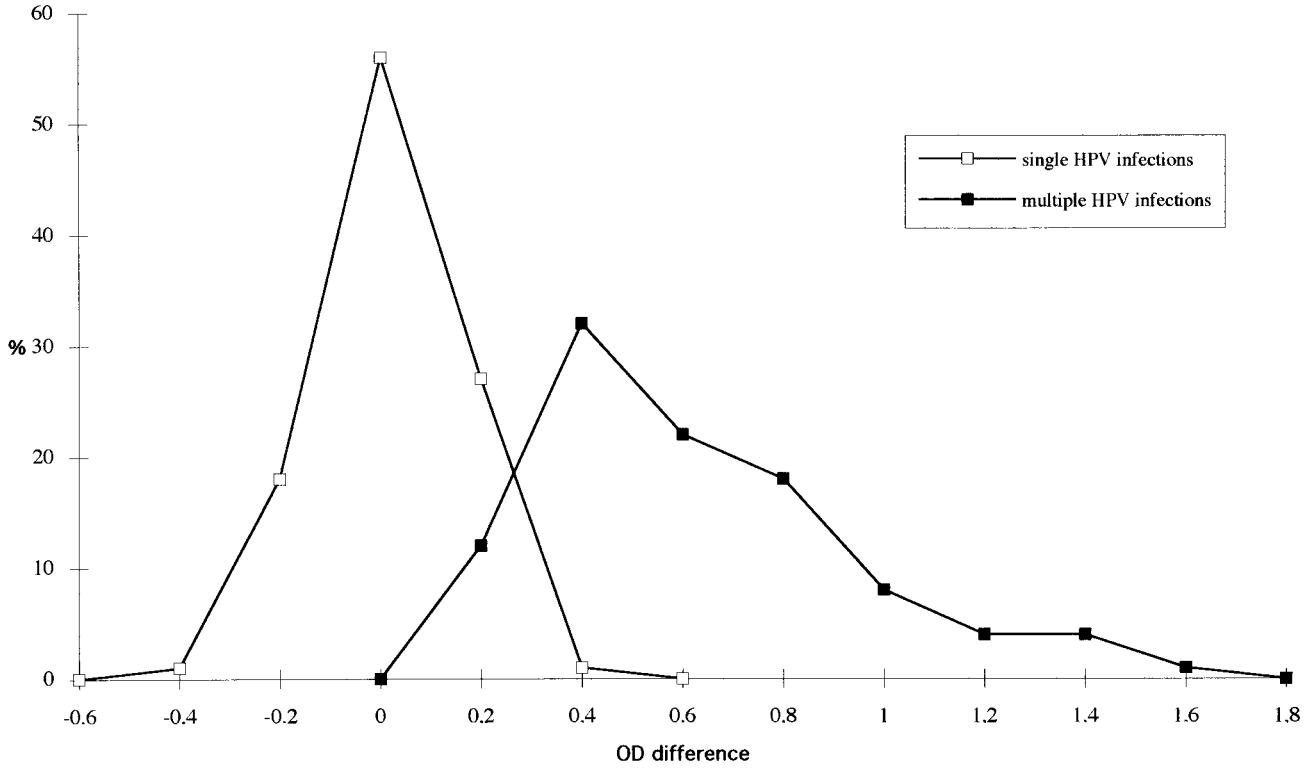

FIG. 2. Histogram of differences between OD values of cocktail probes and individual oligonucleotide probes obtained in PCR-EIA after 1 (n = 33) or 3 (n = 271) h of substrate incubation of all HR- and LR-positive samples. OD differences shown on the x axis represent a range of values from −0.1 to +0.1 OD units of the numbers indicated. On the y axis the percentage of samples is indicated.

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