The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR

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Sequence analysis of human papillomavirus (HPV) general primer GP5/6 mediated PCR products revealed the presence of short highly conserved sequences adjacent to the 3' ends of both primers. Part of these sequences was used to elongate GP5 and GP6 at their 3' ends to generate the primers GP5+ and GP6+, respectively. Compared with the GP5/6 PCR, GP5+/6+ specific PCR on 22 cloned mucosotropic HPVs revealed an improved HPV detection, reflected by a 10- to 100-fold higher sensitivity and a markedly increased signal to background ratio, especially at the gel level. As determined on purified DNA, the sensitivity of this GP5+/6+ based assay was at the femtogram level for those HPV genotypes which match strongly with the primers (e.g. HPV-16) and at the picogram level for HPV types (e.g. HPV-39 and -51) having four or more mismatches with one or both primers. Application of both methods on 264 cervical scrapes of a cohort of women participating in a prospective follow-up study revealed an increase of total HPV positivity from 39% (GP5/6 PCR) to 43% (GP5+/6+ PCR) of the scrapes. Additional HPV typing by PCR specific for the HPV-6, -11, -16, -18, -31 and -33 revealed that all GP5+/6+ PCR positive cases which were negative by GP5/6 PCR (n = 12) contained HPV types different from these six types. These data indicate that the GP5+/6+ PCR method provides an increased detection level mainly of uncommon, apparently poorly matched HPV types in cervical scrapes and most likely in the enlargement of the spectrum of HPVs detectable by this assay.

The human papillomavirus (HPV) group comprises over 70 different epitheliotropic genotypes of which more than 30 are mucosotropic. Approximately one-third of these mucosotropic HPV genotypes have been either isolated from or associated with cervical carcinomas (de Villiers, 1989; Matsukura & Sugase, 1990; Tawheed et al., 1991; Reuter et al., 1991; zur Hausen, 1991). The PCR method has been introduced as the most sensitive method for the detection of HPV DNA in clinical specimens. However, a significant heterogeneity at the nucleotide level is found between the different HPV genotypes. This has hampered the development of a simple universal PCR test for the detection of all HPV genotypes. Despite this, HPV PCR methods have been developed allowing the detection of a broad spectrum of mainly mucosotropic HPV genotypes (Manos et al., 1989; Grégoire et al., 1989; Snijders et al., 1990; Smits et al., 1992; for review see Van den Brule et al., 1993). A combination of the general primers GP5 and GP6, originally selected from the HPV L1 region on the basis of sequence information from HPV-6, -11, -16, -18, -31 and -33 (Snijders et al., 1990), was found to amplify target DNA of at least 27 mucosotropic HPV genotypes under conditions that allow mismatch acceptance (Van den Brule et al., 1990a, 1992; De Roda Husman et al., 1994). The strength of this general primer-mediated PCR (GP-PCR) method has been further substantiated by the detection of HPV DNA in 100% of cervical scrapes cytomorphologically classified as Pap IV (carcinoma in situ) and Pap V (carcinoma) in the Netherlands (Van den Brule et al., 1991; De Roda Husman et al., 1994). This suggests that in the Dutch population all genital high risk HPVs can be detected by this assay.

Still, using GP-PCR in routine diagnostic practice, it has been found that a small number of clinical samples give rise to ambiguous results, reflected by weak GP-PCR signals accompanied with a relatively high background of co-amplified cellular DNA (Van den Brule et al., 1990a). This might be the result of the rather low stringency conditions of primer annealing required for
this assay and may complicate interpretation of screening results. It is presently not known whether the weak signals represent a cross-reaction with cellular sequences or the presence of HPV genotypes having a relatively high number of mismatches with one or both primers and consequently detected at a reduced sensitivity level. To solve this problem attempts were made to improve the sensitivity of the method for poorly matched HPVs and simultaneously to decrease the co-amplification of cellular DNA.

Recently, it has been found that increased primer length contributes to a more efficient amplification probably by increasing the stability of the primer-template complex (Mack & Sninsky, 1988). Additionally, several groups have found that despite the presence of primer-template mismatches, successful amplification by PCR can be ensured by the presence of two to three perfectly matching nucleotides at the 3' primer ends (Newton et al., 1989; Sommer & Tautz, 1989; Evander & Wadell, 1991). Previously, alignment of putative amino acid sequences from the L1 region flanked by both GP5 and GP6 of 24 mucosotropic HPVs has revealed the consensus sequences Thr-Arg-Ser-Thr-Asn (TRSTN) immediately downstream of the GP5 (forward primer) region and Arg-His-X-Glu-Glu (RHXEE) upstream of the GP6 (backward primer) region (Van den Brule et al., 1992). Since these amino acid conservations reflect codon conservations at the nucleotide level we investigated whether elongation of GP5/6 primers with highly conserved sequences at the 3' ends results in an improvement of PCR efficiency. Sequence comparison of the 3' end boundary of the GP5 region of 23 mucosotropic HPV genotypes revealed conserved nucleotides encoding the amino acid sequence Thr-Arg-Ser-Thr-Asn (TRSTN) and the first threonine are included in GP5 (Fig. 1a). For GP5 modification, a T residue (third nucleotide position of the first threonine codon corresponding to HPV 16 sequence) and the first two invariable nucleotides (CA) encoding the first threonine were added to the 3' end of GP5. Further elongation of GP5 was hampered by the fact that the arginine residue can be encoded by six different codons, and consequently both the first and the third nucleotides of this codon are variable. The complementary sequences of the 3' end of the GP6 region revealed nucleotide sequences encoding the amino acid consensus Glu-Glu-Tyr/Ph e, of which the third nucleotide encoding tyrosine/phenylalanine is included in GP6 (Fig. 1b). For GP6 modification, five nucleotides were added to the 3' primer end. In this way the complementary sequences of the two 3' terminal nucleotides of the modified primer represent the invariable bases (GA) encoding glutamic acid. Primer sequences of the elongated 23-mer GP5 (named GP5+) and 25-mer GP6 (named GP6+), aligned with corresponding sequences of the L1 region of 23 mucosotropic HPV genotypes, are indicated in Fig. 1.

The use of the GP5+/6+ primers in the PCR was compared with the original GP5/6 PCR on 1 ng of

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**Fig. 1.** Alignment of the GP5 and GP5+ (a) and GP6 and GP6+ (b) sequences with the corresponding regions in the L1 open reading frame of 23 mucosotropic HPV genotypes, as derived from the EMBL database or kindly provided by Dr H. Delius, Heidelberg, Germany. Partial deduced amino acid sequences are indicated. Dots represent identical bases and characters mismatched base pairs.
of each primer of the GP5/6 or GP5+/6+ primer combination. Each cycle included a 1 min denaturation step at 94 °C, an annealing step to 40 °C for 2 min and a chain elongation step to 72 °C for 1-5 min. The first cycle was preceded by a 4 min denaturation at 94 °C and the last cycle was extended by a 4 min elongation at 72 °C. The GP-PCR products were analysed as previously described (Van den Brule et al., 1990b; Walboomers et al., 1992) by gel electrophoresis, followed by diffusion blotting and low stringgent Southern blot hybridization with a cocktail probe consisting of HPV-6, -11, -16, -18, -31 and -33 specific GP-PCR products. A successful amplification could be determined for all HPV genotypes with both general primer PCR assays. However, only weak bands could be detected after gel electrophoresis of the GP5/6 PCR products of HPV-30, -32, -39, -51 and -66. The reduced signal intensities were accompanied with higher levels of cellular background. The reduction in amplification efficiency of, in particular, HPV-30, -39 and -66 was also evident after low stringency hybridization of the GP5/6 PCR products with the HPV cocktail probe. In contrast, positive signals clearly visible both after gel electrophoresis and hybridization were generated with the elongated primer pair GP5+/6+ for all the types analysed. As shown previously (Snijders et al., 1990), some HPV types (e.g. HPV-18 and -33) exhibited hybridizing fragments of higher molecular mass generated by the GP5/6 system (Fig. 2, lanes HPV-18 and -33). These fragments did not appear using the GP5+/6+ PCR.

The sensitivity of the GP5/6 and GP5+/6+ PCR assays was compared in reconstruction experiments by analysing different concentrations of three cloned HPV DNA's diluted in 100 ng human placental DNA (Fig. 3). The HPVs included types having two and none (HPV 16), four and two (HPV-39) and six and three (HPV-51) mismatches with GP5 and GP6, respectively (see Fig. 1). It appeared that for HPV-16 the sensitivity of the GP5+/6+ PCR was more than 10-fold of that derived from the GP5/6 PCR. After hybridization of the HPV-16 GP5+/6+ products with the HPV cocktail probe a detection level of 1 fg of cloned HPV-16 DNA was reached. This corresponds to an equivalent of about 70 copies of viral genome. After hybridization, the sensitivity of the GP5+/6+ assay for HPV-39 and HPV-51 was more than 100-fold and 10-fold, respectively, compared with the GP5/6 PCR. However, these HPV genotypes showed a reduced sensitivity as compared to HPV-16. Both of these types could be detected at the 10 pg level after low stringency hybridization of the GP5+/6+ PCR products with the HPV cocktail probe. This corresponds to approximately 700 000 viral copies. In all cases the increased sensitivity of the GP5+/6+ PCR was accompanied by a decrease in the amount of co-amplified cellular DNA (Fig. 3).

Subsequently, the GP5/6 and GP5+/6+ PCR
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Table 1. Comparison of GP-PCR methods on cervical scrapes

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<tr>
<th>HPV type</th>
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<tr>
<td></td>
<td>6</td>
</tr>
<tr>
<td>GP5/6 PCR</td>
<td>264</td>
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<tr>
<td>GP5+/6+ PCR</td>
<td>264</td>
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* Multiple infections of HPV-6, -11, -16, -18, -31 and/or -33.
† Types different from HPV-6, -11, -16, -18, -31 and -33.

methods were compared by using them on 264 cervical scrapes collected between January and June 1994 from 222 women participating in a prospective follow-up study to relate HPV presence and morphology of cervical cells with the clinical behaviour of cervical lesions. To perform both HPV detection and cytomorphological analysis, two cervical scrapes were taken. The scrapes were classified according to a slight modification of the Pap procedure as used in the Netherlands (Vooijs, 1987; modified KOPAC classification) and included cases of Pap I (normal cells; n = 120), Pap II (inflammation; n = 73), Pap IIIa (mild and moderate dysplasia; n = 59) and Pap IIIb (severe dysplasia; n = 12). HPV PCR was performed on crude cell suspensions after processing the scrapes by a freeze and thawing method essentially as described before (Van den Brule et al., 1990b; 1991; Walboomers et al., 1992). A 10 µl processed sample taken out of a 1 ml cell suspension was used for each PCR assay. All samples appeared positive after prescreening by PCR using β-globin specific primers (Saiki et al., 1985), indicating a proper quality of the samples. Of the 264 cervical scrapes analysed, 114 scrapes (43%) gave positivity with the GP5+/6+ PCR, whereas 102 positive scrapes (39%) were scored with the GP5/6 PCR (Table 1). In addition to this discrepancy, a difference was also evident between the number of HPV specific amplification signals already visible at the gel level. The GP5+/6+ assay revealed a clear signal at the gel level for 71 cases (27%), whereas in the GP5/6 PCR only 42 cases (16%) gave a signal distinguishable at the gel level (data not shown). Further typing of GP-PCR positive samples by type-specific PCR for HPV-6, -11, -16, -18, -31 and -33, performed as described by Van den Brule et al. (1990b), revealed that the difference in HPV positivity scored by both assays was due to an enhanced GP5+/6+ PCR detection of HPV types different from these six common genital HPVs (tentatively named HPV X; Table 1). With the GP5+/6+ PCR 48 cases of HPV X were scored as compared to 36 HPV X cases detected by the GP5/6 PCR.

These data together indicate that elongation of the GP5/6 primers with three and five nucleotides, respectively, results in an enhanced HPV detection level. Sequence comparison of 23 mucosotropic HPV genotypes initially led to the selection of the triplet CAC as the most favourable 3' end extension of GP5, since this is the most common sequence amongst the mucosotropic HPV types (Fig. 1). This, however, gave rise to a decrease in PCR efficiency which was most likely due to the introduction of an internal complementary sequence of six nucleotides (5' TTT GTT ACT GTG GTA GAT ACC 3'). This sequence might enhance the formation of primer dimers interfering with the efficiency of amplification of the target DNA. However, the modified GP5 with the TAC extension at the 3' end appeared to be a very efficient forward general primer. On the other hand, incorporation of the sequence 5' TACTC 3' instead of 5' TATTC 3' at the 3' end of GP6 gave the same results (data not shown). Although the sequence conservation at the 3' end of GP6 would have allowed GP6 modification
by an extension of seven nucleotides, this was omitted to provide the modified GP6 primer with nearly the same $T_m$ value as the modified GP5 primer. Despite the fact that the $T_m$ value of both modified primers was increased, the performance of the PCR at higher stringency conditions by raising the annealing temperature revealed a lower amplification efficiency for poorly matched types. Obviously, the principle of mismatch acceptance requires low stringency annealing, which does not interfere dramatically with the novel assay given the low background. Incorporation of degenerate bases at the ambiguous positions 18 and 21 in the case of GP5+ and positions 21 and 23 in GP6+ to render them more identical to the majority of sequenced HPVs, did not give rise to improved PCR efficiency under the conditions used. This indicates that, at least to a certain extent, low stringency conditions of PCR efficiently compensate for primer/template mismatches not present at the extreme 3′ primer ends.

Despite the fact that for the majority of the HPV types the 3′ end extension in GP5+ resulted in the introduction of one more primer–template mismatch (see Fig. 1a), the use of GP5+/6+ in the PCR showed an enhancement rather than a reduction in PCR efficiency, as was most evident for HPV-30 (Fig. 2, lanes HPV-30). Furthermore, the sensitivity test revealed that for HPV-39 and -51 a more efficient amplification was obtained with the GP5+/6+ combination, despite the fact that the extension in GP6+ introduced one more mismatch with these types. Consequently, primer extension may in part overcome PCR reduction due to mismatches. Still, the number of primer–template mismatch appeared critical for the PCR as shown in the sensitivity test. HPV-16, having a maximum of two mismatches with one primer was amplified at a significantly higher efficiency than HPV-39 and -51, having four and six mismatches with one of the primers, respectively.

After hybridization with the cocktail probe, application of the GP5+/6+ PCR method on cervical scrapes revealed an increase of total HPV positivity of 4% compared with the value obtained with the original GP5/6 PCR assay. This is markedly lower than the differences in HPV positivity (11%) found after judgement at the gel level. This indicates that the use of the modified method on cervical scrapes particularly results in the improved detection of HPV DNA already detectable by the original assay, rather than by exhibiting large numbers of additional HPV positive cases. Surprisingly, the GP5+/6+ positive cases which were negative by GP5/6 PCR did not include any of the six common genital types, despite the fact that also for these types the modified method is at least 10-fold more sensitive, as determined for HPV-16. Obviously, the sensitivity for these types, which all can be considered strongly matched types (Fig. 1), exceeds the number of viral copies generally found even in Pap I smears. Apparently, the additional GP5+/6+ positivity includes poorly matched types amongst genital HPVs like HPV-39 and HPV-52. This strong contrast can be expected since a 10000-fold difference in GP-PCR sensitivity was observed between strongly matched and poorly matched genital HPV types. Although the additional GP5+/6+ PCR positive cases include scrapes of all Pap classes (Pap I to Pap IIIb) analysed, the clinical significance of detecting these types still needs to be determined. This is currently under investigation in the follow-up study and will be accompanied by typing these HPV Xs. It should be considered that despite the fact that with the novel method no severe increase of HPV positivity was scored in this cohort study, this might be different for other cohorts of women (e.g. other geographical regions). Furthermore, the method could be of great value to determine HPV prevalence in extragenital sites, which can be suspected to be associated with a different spectrum of HPVs.

Due to the increased signal to background ratio the GP5+/6+ PCR offers possibilities to alter the final detection system to avoid the laborious gel electrophoresis step. In combination with properly selected non-radioactively labelled probes this would enable detection by ELISA based formats.

Finally, the finding that these elongated primers generate HPV Xs visible at the gel level means that cloning and characterization of these products by sequencing will be more easy to perform.

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