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Comprehensive Study of Several General and Type-Specific Primer Pairs for Detection of Human Papillomavirus DNA by PCR in Paraffin-Embedded Cervical Carcinomas

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We have compared the efficacies of three general primer pairs for the detection of human papillomavirus (HPV) DNA in formaldehyde-fixed paraffin-embedded carcinomas. The use of these primer pairs leads to underestimates of the HPV prevalence (GP5/6, 61.1%; CPI/IIG, 57.4%; MY09/11, 46.9%; combined, 72.8%). The efficacy of each primer pair seemed to be inversely correlated to the length of the amplimer produced. By using newly developed type-specific primer pairs (amplimer length, approximately 100 bp), an increase in HPV DNA detection (87.6%) was found.

There is strong evidence to link the presence of human papillomaviruses (HPV) to the development of cervical cancer (11). The presence of HPV DNA in over 90% of carcinomas of the uterine cervix has been established by PCR (12, 18, 20). Ideally, one primer pair would suffice for PCR amplification of all mucosatropic HPV types (6, 9, 14, 16, 20). As paraffin-embedded material is the main source for retrospective studies using PCR, the efficacies of three general primer systems with archival tissues were investigated, along with those of HPV type-specific (TS) primer pairs and sequence analysis.

Paraffin-embedded tissues from 162 women treated for squamous cell cervical carcinomas at the Department of Obstetrics and Gynaecology, University Hospital, Groningen, The Netherlands, were available. DNA was extracted from 10-μm sections by deparaffination and digestion with proteinase K, as described by Wright and Manos (19). Adjacent sections were hematoxylin and eosin stained and assessed for the presence of tumor cells.

All oligonucleotide primers were obtained from Pharmacia, Woerden, The Netherlands. The primers used were the MY09-MY11 set (MY09/11) (9), the GP5-GP6 set (GP5/6) (14), and the CP-I–CP-IIG set (CPI/IIG) (16) (Table 1). As a control for DNA extraction, β-globin primers PC03 and PC04 (13) were used. Conventional TS primers were used for HPV type 16 (HPV-16), -18, -31, and -33 (4, 10, 18). In addition, for the detection of HPV-16, -18, -31, and -33 DNAs, supplementary primer pairs (new TS primers) spanning shorter DNA sequences were constructed (Table 2). PCR amplification was performed by the method of Saiki et al. (13). The final 100-μl PCR mixture contained a 10-μl sample, 10 mM Tris-HCl (pH

TABLE 1. Specifications of oligonucleotides used as primers and probes for general HPV detection by PCR

Primer or probe	Sequence (5′–3′)[a]	Target[b]	Amplimer length (bp)	Reference
Primers				
MY09	CGTCCMARRGGAWACTGATC	L1	452	9
MY11	GCMCAGGGWCATAAYAATGG			
GP5[c]	TTTGTTACTGTGGTAGATAC	L1	155	14
GP6	ACTAAATGTCAAATAAAAAG			
CP-I	TTATCAWATGCCCAYTGTACCAT	E1	188	16
CP-IIG	ATGTTAATWSAGCCWCCAAAATT			
PC03	ACACAACTGTGTTCACTAGC	β-Globin		13
PC04	CAACTTCATCCACGTTCACC			
Probes[d]				
GP1	CTGTTGTTGATACTACACGCAGTAC			17
GP2	CTGTGGTAGATACCACWCGCAGTAC			17
PR-G	AGCAYTRTATTGGTATMGAACAGG			16

[a] M, A+C; R, A+G; S, G+C; W, A+T; Y, C+T.
[b] L1, HPV late structural protein 1; E1, HPV early protein 1.
[c] Used in combination with random prime-labelled probe GP5/6.
[d] GP1 and GP2 are general probes used with MY09/11, and PR-G is a probe for genital HPV used with CPI/IIG.

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TABLE 2. Specifications of oligonucleotides used as primers and probes for TS HPV detection by PCR

Primer pair or probe	Sequence(s) (5′–3′)[a]	Amplimer length (bp)	Reference
Primer pairs			
Conventional TS 16	GTGTGTACTGCAAGCAACAG, GCAATGTAGGTGTATCTCCA	395	4
New TS 16	GGTCGGTGGACCGGTCGATG, GCAATGTAGGTGTATCTCCA	96	This study
Conventional TS 18	AAGGATGCTGCACCGGCTGA, CACGCACACGCTTGGCAGGT	217	10
New TS 18	CCTTGGACGTAAATTTTTGG, CACGCACACGCTTGGCAGGT	115	This study
Conventional TS 31	ATGGTGATGTACACAACACC, GTAGTTGAGGACAACTGAC	514	18
New TS 31	GGGATTGTTACAAAGCTACC, CGCTTAGTAGACGTCGTCGC	110	This study
Conventional TS 33	ATGATAGATGATGTAACGCC, GCACACTCCATGCGTATCAG	506	10
New TS 33	CCACCACTGCTTCTTACCTC, ACCATTTTCATCAAATGGGA	114	This study
Probes			
TS 16[b]	CAAGAACACGTAGAGAAACCCAGCTGTAAT		4
TS 18	TGGTTCAGGCTGGATTGCGTCGCAAGCCCA		10
TS 31	ACCTGCGCCTTGGGCACCAGTGAAGGTGTG		18
TS 33	CAAATGCAGGCACAGACTCTAGATGGCCAT		10

[a] For primer pairs, the sequences are given in the order of the 5′ primer followed by the 3′ primer.
[b] The new TS 16 primers were used in combination with random prime-labelled HPV-16 E6 probe.

9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin, 200 μM deoxynucleoside triphosphates, 50 pmol of each primer, and 0.25 U of SuperTaq (Sphaero Q, Cambridge, United Kingdom), except for the MY09/11 PCR, in which 0.5 U of SuperTaq was used. The standard PCR conditions were 1 min at 95°C, 1 min at the annealing temperature, and 2 min at 72°C for 40 cycles. After PCR, 25 to 30 μl of all samples was run on a 2% agarose gel; gels were blotted (Hybond N^+; Amersham, Little Chalfont, United Kingdom) and hybridized with the appropriate probe (Tables 1 and 2). Blots were washed twice for 15 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate. For all samples positive in general PCRs and negative in both TS PCRs, the general PCR amplimer bands were sequenced by the protocol of the manufacturer of the T7 sequencing kit (Pharmacia).

All patients were positive in the β-globin PCR (100-bp DNA fragment). Of 162 carcinomas, 118 (72.8%) were positive for HPV DNA; 99 (61.1%) were positive with GP5/6, 93 (57.4%) were positive with CPI/IIG, and 76 (46.9%) were positive with MY09/11 (Table 3). All tissue samples were also tested with conventional TS primer pairs. Of 118 carcinomas positive with one or more general primer pairs, 59 carcinomas were positive with conventional TS primers; the remaining 59 carcinomas were still untypeable. None of the tissue samples negative with general primers were positive with conventional TS primer pairs. Further evaluation was performed with newly selected TS primer pairs. By using these new TS primer pairs, 43 of the 59 untypeable carcinomas could be typed. Furthermore, with these new TS primer pairs, 24 of the 44 carcinomas negative with all general HPV primer pairs were positive for HPV (19 contained HPV-16 DNA and 5 contained HPV-18 DNA), thus raising total HPV positivity to 87.6% for this group of 162 cervical carcinomas. In this way, 95 carcinomas were found to contain HPV-16 DNA, 23 contained HPV-18 DNA, 2 contained HPV-31 DNA, 5 contained HPV-33 DNA, and 1 carcinoma was positive for both HPV-16 and -31.

Sixteen carcinomas positive with general primers were still untypeable; of these, 14 were subjected to sequencing. From two patients, insufficient amounts of material were available for sequencing. Other HPV types found in this way were HPV-6, HPV-34 (twice), HPV-45, HPV-52, HPV-58, HPV-X (some similarity to HPV-34 [data not shown]), and HPV-X (some similarity to HPV-18, not reacting with the conventional or new HPV-18-specific primer pair [data not shown]). Five carcinomas were HPV-16 positive, and one was HPV-33 positive; however, they did not react with conventional or new TS primers.

The results described above show that the use of one general HPV primer pair with paraffin-embedded tissues grossly underestimates the presence of HPV DNA in the studied population. Moreover, even the combination of the three most widely used primer pairs, MY09/11, GP5/6, and CPI/IIG, still results in an underestimation of HPV prevalence.

Several authors have reported difficulties in reproducing PCR results with formaldehyde-fixed tissues (1, 2, 5, 15). Recently, the antagonistic effect of formaldehyde fixation due to DNA modification, causing the inhibition of PCR amplification of longer (>200-bp) amplimers, has been described by Karlsen et al. (7).

Our results show that the efficiency of the primer pair is inversely correlated to the length of the amplimer and that the amplification of a PCR product of less than 200 bp is also affected by formaldehyde fixation, as the GP5/6 (amplimer length, 155 bp; positivity, 61.1%) and CPI/IIG (188 bp and 57.4%, respectively) primer pairs do not reach the overall positivity of 87.6%.

The newly designed TS primers proved to be of great value, since 72.9% of the general primer-positive untypeable carcinomas could be typed. Furthermore, with the new TS primers, 24 of the 44 general HPV PCR-negative carcinomas were found to contain HPV DNA.

TABLE 3. HPV PCR-positive results of 162 cervical carcinomas with three general primer sets

PCR result with primer set[a]			No. with pattern
GP5/6	CPI/IIG	MY09/11	
−	−	−	44
+	−	−	11
−	+	−	8
−	−	+	4
+	+	−	23
+	−	+	10
−	+	+	7
+	+	+	55

[a] Of 162 carcinomas, 118 (72.8%) were positive with at least one primer set. The total numbers detected by primer sets GP5/6, CPI/IIG, and MY09/11 were 99 (61.1%), 93 (57.4%), and 76 (46.9%), respectively.

It can be argued, however, that the amplification of these 100-bp amplimers is still hampered by formaldehyde fixation, as studies using fresh tissue samples commonly report HPV prevalences well over 90% (8, 12, 18, 20). Alternatively, the HPVs not detected by the TS primers are types other than HPV-16, -18, -31, and -33.

In conclusion, general primer pairs are unsatisfactory for the amplification of HPV DNA from formaldehyde-fixed paraffin-embedded tissue samples. Therefore, we propose the following strategy for cervical carcinomas: amplification with new TS primers (giving approximately 100-bp products), amplification of TS-negative tissues with CPI/IIG, GP5/6, and/or MY09/11 primer pairs, and subsequent sequencing to detect other HPV types. At this moment, this should lead to the highest possible detection of HPV DNA from formaldehyde-fixed paraffin-embedded carcinomas.

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