JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2007, p. 2537–2544 Vol. 45, No. 8
0095-1137/07/$08.00+0 doi:10.1128/JCM.00747-07

Development of a Sensitive and Specific Multiplex PCR Method Combined with DNA Microarray Primer Extension To Detect Betapapillomavirus Types▽

Tarik Gheit,[1] Gaëlle Billoud,[1] Maurits N. C. de Koning,[2] Federica Gemignani,[3] Ola Forslund,[5] Bakary S. Sylla,[1] Salvatore Vaccarella,[1] Silvia Franceschi,[1] Stefano Landi,[3] Wim G. V. Quint,[2] Federico Canzian,[4] and Massimo Tommasino[1]*

International Agency for Research on Cancer, Lyon, France[1]; DDL Diagnostic Laboratory, 2275 CX Voorburg, The Netherlands[2]; Genetica, Dipartmento di Scienze Uomo e Ambiente, University of Pisa, Pisa, Italy[3]; Deutsches Krebsforschungszentrum, D-69120 Heidelberg, Germany[4]; and Department of Medical Microbiology, Malmö University Hospital, Lund University, S-205 02 Malmö, Sweden[5]

Received 6 April 2007/Returned for modification 12 May 2007/Accepted 10 June 2007

Emerging lines of evidence indicate that the cutaneous human papillomavirus (HPV) types that belong to the genus *Betapapillomavirus* (beta HPV) are involved in the development of nonmelanoma skin cancer. Unlike the situation for mucosal HPV types, highly sensitive and reliable methods to identify characterized cutaneous HPV types in a single assay are limited. Here, we describe a novel one-shot method for the detection of all characterized beta HPV types, namely, HPV type 5 (HPV5), 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 49, 75, 76, 80, 92, 93, and 96. This assay combines two different techniques: multiplex PCR using HPV type-specific primers for amplification of each E7 gene and array primer extension (APEX) for typing. This method has been validated using clinical samples which were analyzed simultaneously for the presence of cutaneous HPV types by two additional methods, i.e., the FAP59/64 PCR protocol and a commercially available PCR-reverse hybridization assay (PM-PCR RHA). Our data show good agreement between the results obtained with the multiplex PCR/APEX assay and the PM-PCR RHA method (overall HPV positivity of 92.2% for multiplex PCR/APEX assay versus 90.6% with the PM-PCR RHA) (kappa value, 50; 95% confidence interval, 13 to 88). In addition, the multiplex PCR/APEX assay showed higher sensitivity than the PM-PCR RHA did. This favorable feature and the high-throughput potential make this assay ideal for large-scale clinical and epidemiological studies aimed at determining the spectrum of cutaneous types in skin cancer.

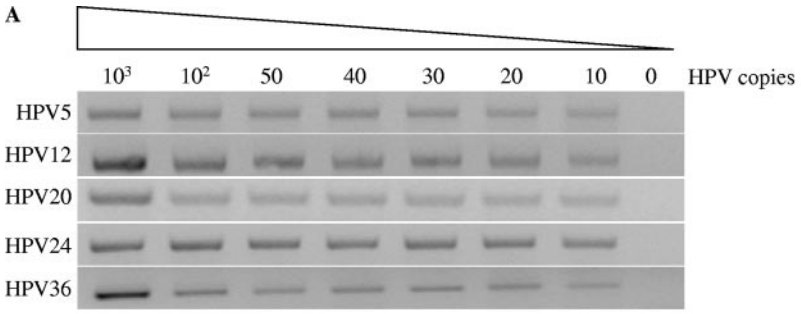
The family of the epitheliotropic human papillomaviruses (HPVs) comprises approximately 100 different types that have been subgrouped into different genera according to their genomic DNA sequence (9). In addition, the HPVs can be subdivided into mucosal and cutaneous HPVs based on their tissue tropism. Members of the genus *Alphapapillomavirus*, referred to as mucosal high-risk HPV types, have been clearly linked to cervical cancer (23). Emerging lines of evidence indicate that another group of HPVs that belong to the genus *Betapapillomavirus* (beta HPV) may also be involved in human carcinogenesis, i.e., nonmelanoma skin cancer (NMSC) (reviewed in reference 16). They were first isolated in skin cancer-prone patients suffering from a rare autosomal recessive genetic disorder called epidermodysplasia verruciformis, but it is now clear that they are commonly found in the skin of healthy individuals (16). Twenty-five beta HPV types have been identified and sequenced so far, namely, HPV type 5 (HPV5), 8, 9, 12, 14, 15, 17, 19 to 25, 36 to 38, 47, 49, 75, 76, 80, 92, 93, and 96. However, with the development of more sensitive HPV detection methods, it is now evident that many additional beta HPV types exist.

Several methods have been developed for the detection of cutaneous HPV types. Shamanin et al. (19, 20) described the degenerate primer pair, HPV2/B5, to detect HPVs from several genera with the exception of the phylogenetic clade comprising HPV4, HPV48, HPV50, HPV60, and HPV65, for which the primer pair F14/B15 was used (1). A nested PCR-based protocol implies the use of several primer sets and detects beta and non-beta cutaneous HPV types (5). Forslund et al. have designed a single pair of degenerate PCR primers in the two relatively conserved regions of the L1 open reading frame that can detect a large spectrum of cutaneous HPV types (10). The limitation of the methods described above is that the identification of the HPV types is acquired by DNA sequencing of the PCR amplimers directly or after cloning. This procedure is laborious and, in the case of multiple infections, may not allow the identification of all HPV types present in the skin specimen. Two new methods have recently been described that are still based on the use of consensus/degenerate primers, but the HPV typing is achieved by reverse line blotting (7) or a reverse hybridization assay (RHA) (8).

All these methods have been used successfully to detect cutaneous HPV DNA in skin specimens from immunocompetent and immunosuppressed individuals (reviewed in references 6 and 16). However, there is no consensus on the prevalence and spectrum of HPV types in skin tumors across studies. The difference in the prevalence rate and diversity of HPV types detected in skin lesions in various studies may

* Corresponding author. Mailing address: Infections and Cancer Biology Group, International Agency for Research on Cancer, 150, cours Albert-Thomas, 69372 Lyon, France. Phone: 33-4-72738191. Fax: 33-4-72738442. E-mail: tommasino@iarc.fr.

▽ Published ahead of print on 20 June 2007.



B

N		HPV5_1	N
A		HPV5_2	C
HPV8_1	HPV9_1	HPV12_1	HPV14_1
HPV8_2	HPV9_2	HPV12_2	HPV14_2
HPV15_1	HPV17_1	HPV19_1	HPV20_1
HPV15_2	HPV17_2	HPV19_2	HPV20_2
HPV21_1	HPV22_1	HPV23_1	HPV24_1
HPV21_2	HPV22_2	HPV23_2	HPV24_2
HPV25_1	HPV36_1	HPV37_1	HPV38_1
HPV25_2	HPV36_2	HPV37_2	HPV38_2
HPV47_1	HPV49_1	HPV75_1	HPV76_1
HPV47_2	HPV49_2	HPV75_2	HPV76_2
HPV80_1	HPV92_1	HPV93_1	HPV96_1
HPV80_2	HPV92_2	HPV93_2	HPV96_2
G	BGlo_1		U
N	BGlo_2		N

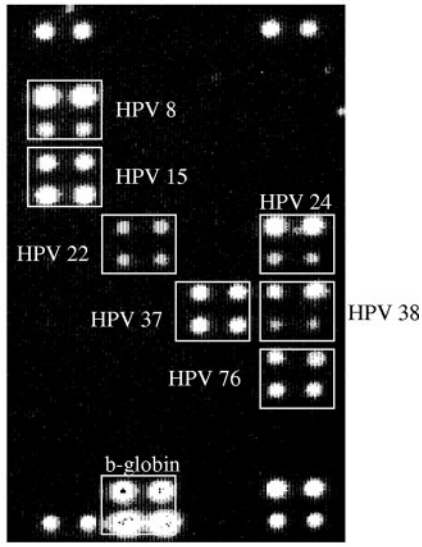


FIG. 1. HPV typing by multiplex PCR/APEX assay. (A) Multiplex PCR. Reactions were performed using serial dilutions of cloned HPV5, 12, 20, 24, or 36 plasmids (1,000 to 0 copies) mixed with 50 ng of genomic DNA of C33A cells. (B) Layout of the APEX chip. The oligonucleotides printed on the array are indicated in the boxes. Their sequences are given in Table 2. Each oligonucleotide has been spotted in duplicate. The four corners contain a mixture of four self-elongating marker oligonucleotides that give signals in all dideoxynucleotide channels (named N) and one self-elongation marker oligonucleotide that gives a signal only in one dideoxynucleotide channel (A, G, C, or U). (C) APEX assay of the multiplex PCR. After fragmentation of the PCR product, APEX was performed as described in Materials and Methods. The image was obtained after excitation by a laser wavelength corresponding to cyanine 5 bound to ddUTP. The positive signals for HPV8, 15, 21, 24, 37, 38, 76, and β-globin (b-globin) correspond to four white spots (two different oligonucleotides for each type spotted in duplicate) and are highlighted by the white boxes.

reflect the use of different PCR-based detection methods (22). The use of degenerate and/or consensus primers offers the advantage of detecting a large spectrum of HPV types by a single PCR. However, the primers may be afflicted with decreased sensitivity for certain HPV types. This aspect is particularly important in the case of multiple infections, which appear to be extremely frequent in skin (3, 17).

In this article, we describe the development of a novel PCR-based assay for the detection of all beta HPV types, which use type-specific primers designed to amplify the E7 gene.

MATERIALS AND METHODS

Development of multiplex PCR primers. Complete or partial HPV sequences were obtained from GenBank and used for alignment of the whole E7 gene. This region is highly divergent, and primers specific to each type were developed based on the more conserved parts of this gene. The E7 genes of the following accession numbers of GenBank were used as references: NC_001531 (HPV5), M12737 (HPV8), NC_001596 (HPV9), X74466 (HPV12), D50546 (HPV14), X74468 (HPV15), X74469 (HPV17), X74470 (HPV19), U31778 (HPV20), U31779 (HPV21), U31780 (HPV22), U31781 (HPV23), U31782 (HPV24), X74471 (HPV25), U31785 (HPV36), U31786 (HPV37), M32305 (HPV47), NC_001591 (HPV49), U31787 (HPV38), Y15173 (HPV75), Y15174 (HPV76), Y15176 (HPV80), NC_004500 (HPV candidate type 92), AY382778 (HPV93), and AY382779 (HPV candidate type 96). Oligonucleotides were synthesized by MWG Biotech (Ebersberg, Germany) and mixed to obtain a 10× solution containing 2 μM of each primer. Two primers for the amplification of β-globin (GenBank accession number AY260740) were added to provide a positive control for quality of the template DNA (18). Some primers are used for more than one type (e.g., HPV75 forward [F]/HPV76 F) due to the high similarities between the E7 genes. Two different pairs of primers were designed for HPV5, since the first pair (5-1 primers) did not efficiently amplify the E7 region. The second pair (5-2 primers) amplified a shorter fragment (186 bp). Since the 5-1 F primer was also used to amplify HPV36 E7, a new forward primer was added for this HPV type (36-2 F). 5-1 primers were used for the HPV typing shown in Tables 3 and 4, while 5-2 and 36-2 F primers were used in the experiment showed in Table 5.

Multiplex PCR conditions. PCRs were performed with the QIAGEN Multiplex PCR kit according to the manufacturer's instructions. dUTP (Fermentas) was added to a final concentration of 50 μM to allow PCR product fragmentation (see below). PCR products ranged in size from 186 bp to 280 bp. The presence and size of PCR products were checked systematically on agarose gels.

Generation of the DNA chip. Two oligonucleotides covering two 30-bp regions of each E7 gene and containing a chain of six carbon atoms at the 5′ end (C-6 oligonucleotides) were designed (see Table 2), synthesized by MWG Biotech (Ebersberg, Germany) and spotted onto silanized slides, as reported elsewhere (4, 14). The layout of the chip is shown in Fig. 1B.

In order to have all immobilized C-6 oligonucleotides labeled with only one dye-labeled dideoxynucleoside triphosphate terminator after the primer extension, we designed each oligonucleotide in a E7 region immediately adjacent to a T at the 3′ end. In this way, after hybridization of the PCR products on the chip, only uracyl (cyanine 5-ddUTP) is incorporated during the sequence-specific extension reaction of the 3′ ends of the oligonucleotides. Thus, the analysis of the chip can be performed on very simple instruments equipped with only one laser.

APEX assay. Following the protocol previously described (12), PCR products were purified, concentrated using Millipore Y30 columns, and fragmented to facilitate the hybridization reaction with the arrayed oligonucleotides. Fragmentation was achieved by treatment of purified PCR products with 1 U uracil *N*-glycosylase (Epicenter Technologies, Madison, WI) and 1 U shrimp alkaline phosphatase (Amersham Biosciences, Milwaukee, WI). The mixture was incubated at 37°C for 1.5 h and at 95°C for 30 min. Fragmented PCR products were added to a reaction mixture containing cyanine 5-ddUTP and 4 U of Thermo Sequenase (Amersham Biosciences, Uppsala, Sweden) and placed onto the chip and incubated at 58°C for 10 min. After hybridization of the PCR products to the chip, the extension reaction was performed to allow incorporation of cyanine 5-ddUTP. Slides were washed to remove the traces of nonhybridized PCR products and cyanine 5-ddUTP not incorporated. Slides were imaged by a Genorama-003 four-color detector (Asper Biotech, Tartu, Estonia). The signal for specific HPV types in the APEX method was considered positive only if both APEX probes gave a signal in the cyanine 5 channel. Fluorescence intensities were measured and converted to base calls according to the Genorama image analysis and genotyping software (Asper Biotech, Tartu, Estonia). To ensure quality control, each APEX oligonucleotide was spotted in duplicate.

Validation of the multiplex PCR/APEX assay. To evaluate the sensitivity and specificity of the multiplex PCR/APEX typing method, we analyzed two series of test samples: artificial mixtures containing cloned HPV genomes at different relative concentrations and human skin specimens. The latter comprised swabs of squamous cell carcinomas ($n = 9$), basal cell carcinomas ($n = 25$), keratoacanthomas ($n = 3$), actinic keratoses ($n = 12$), seborrheic keratoses ($n = 10$), and other benign lesions ($n = 5$) collected at the University Hospital, Malmö, Sweden. For collection of superficial cells, a cotton tip was prewetted in saline (0.9% NaCl), rolled on the lesion (within margins of the lesion), and suspended in 1 ml of saline. For each swab sample, 10 μl of the 1-ml suspension was added directly to the PCR mixture without DNA extraction.

To validate the multiplex PCR/APEX, HPV typing was performed independently in three different laboratories by three different methods: (i) multiplex PCR/APEX (Lyon, France); (ii) FAP59/64 PCR protocol, followed by cloning and sequencing of the PCR products (Malmö, Sweden) (10); and (iii) PM-PCR RHA method (skin β HPV prototype research assay; Diassay BV, The Netherlands) (8).

In some cases purified amplicons were sequenced with the respective amplification primers on an ABI 3700 automated DNA analysis platform to confirm the results obtained with the multiplex PCR/APEX assay.

Statistical methods. Descriptive statistics were carried out for the 64 skin samples tested for HPV detection with the Multiplex PCR/APEX method, the PM-PCR RHA method (8), and the FAP59/64 PCR protocol, followed by cloning and sequencing of the PCR products (10). The overall and type-specific percentages of HPV positivity and the overall number of infections were calculated for each detection method.

In order to evaluate the reproducibility of the methods, testing was repeated in a blind manner three times in 10 subjects (only for Multiplex PCR/APEX and PM-PCR RHA methods). The percent of positive agreement was defined as the ratio between the sum of the number of HPV types detected by all three measurements and the sum of the number of different HPV types detected by any of the three measurements.

The kappa statistic was calculated to measure the agreement between overall HPV positivity detected by the two methods (Multiplex PCR/APEX and PM-PCR RHA methods) beyond that expected by chance. Kappa estimates of less than 0.2 represent "poor" agreement, while for kappa values between 0.2 and 0.6, the agreement can be considered "fair" to "moderate," and kappa values of more than 0.6 represent "good" or "very good" agreement (2).

TABLE 1. Sequences of forward and reverse HPV type-specific primers and size of the PCR-amplified fragments[a]

HPV type	Primer direction[b]	Primer sequence	Size of PCR fragment (bp)
5-1	F	5′-GAGGTCACCGTGCAAGATATTRTTC-3′	269
	R	5′-ACAGTCAGGRCACAGGAGCTGC-3′	
5-2	F	5′-AGTGAGGTGCAGCCCGAAGTGCTA-3′	186
	R	5′-CTCTAATACCAAATTCTGTGGCGTG-3′	
8	F	5′-CACTGTGCAAGATTTTGTGTTGAAG-3′	264
	R	5′-GCACTCAGGACACAGAAGCTGTAG-3′	
9	F	5′-GCTACTATACCAGAGGTGGTTCT-3′	241
	R	5′-CACTCCGGACACACCAGTTGTATATC-3′	
12	F	5′-CACCGTGCAAGATTTTACCTTGGAG-3′	280
	R	5′-GTTTGCAGTTMCCACGGCACTCTG-3′	
14	F	5′-GAGGTCACATTGCAAGATATTGTTC-3′	266
	R	5′-GCACTCAGGACACAACAGCTGC-3′	
15	F	5′-GAAGCTACTATACCAGATATAGWGC-3′	241
	R	5′-CCTGGACACACCAACTTAACTTC-3′	
17	F	5′-GAAGCTACAATACCAGAWATAGTGC-3′	248
	R	5′-GTTAGGACACACCAACTGTACTTC-3′	
19	F	5′-GGTAAAGAGGTGATATTGCAAGAC-3′	269
	R	5′-CTCCGGGCACAACAGTTGCAATTC-3′	
20	F	5′-GAGGTCACATTGCAAGATATTGTG-3′	265
	R	5′-CAGTCAGGACACAACAGCTGCAG-3′	
21	F	5′-GAGGTCACATTGCAAGATATTGTTC-3′	263
	R	5′-GCACTCAGGACACAACAGCTGC-3′	
22	F	5′-GCTACTCTGTGTGATATAGTTCTTG-3′	258
	R	5′-GACAGGTGGGACACAAAAGTTTTAC-3′	
23	F	5′-CAAGCTACTCTTCGTGATATAGTTC-3′	251
	R	5′-ACAGGCAGGACACACCAGCTTCAC-3′	
24	F	5′-GAGGTCACTCTACAAGACATTGTC-3′	245
	R	5′-GCAGTCGGGACACAAAATGACCACC-3′	
25	F	5′-GGAGGTCACATTGCAAGATTTTAC-3′	267
	R	5′-ACACTCCGGACACAACAGCTGCAG-3′	
36-1	F	5′-GAGGTCACCGTGCAAGATATTRTTC-3′	266
	R	5′-ACACTCGGGACACAGAAGCTGCAG-3′	
36-2	F	5′-GGAATTACCAAACGAGGATACGG-3′	186
37	F	5′-GAAGCTACAATACCAGAWATAGTGC-3′	245
	R	5′-GTTTGGACAAACCAGCTGCACTTC-3′	
38	F	5′-CAAGCTACTCTTCGTGATATAGTTC-3′	259
	R	5′-CAGGTGGGACACAGAAGCCTTAC-3′	
47	F	5′-GGTCACCGTGCGAGATATTGTTC-3′	283
	R	5′-GTTTGCAGTTMCCACGGCACTCTG-3′	
49	F	5′-GATTGGGAAAGAAGTTACAATACCAG-3′	278
	R	5′-CACTGAGGACACAAGAATTGCAG-3′	
75	F	5′-GGCTACATTACCTGACATTGTGC-3′	262
	R	5′-GACACTCMGGACACAGGAATTGC-3′	
76	F	5′-GGCTACATTACCTGACATTGTGC-3′	262
	R	5′-GACACTCMGGACACAGGAATTGC-3′	
80	F	5′-GAAGCTACTATACCAGATATAGWGC-3′	241
	R	5′-CCTGGACACACCAACTTAACTTC-3′	
92	F	5′-CAGGCTACTATACCAGATATTGTGC-3′	247
	R	5′-CAGTCAGGACACAGCAACGCTAT-3′	
93	F	5′-GAGGTCACTCTGCAAGACATTGTG-3′	248
	R	5′-GCAGTTAGGACACAGAAGATCCAAC-3′	
96	F	5′-GCAACTATACCAGACATAAAGCTGG-3′	268
	R	5′-CAAGCAGGACACAGCAAAGAAGG-3′	

[a] Due to the homology in the E7 gene of different HPV types, the following primers have identical sequences: HPV5 F/HPV36 F (version 1), HPV14 F/HPV21 F, HPV15 F/HPV80 F, HPV17 F/HPV37 F, HPV23 F/HPV38 F, HPV75 F/HPV76 F, HPV12 R/HPV47 R, HPV14 R/HPV21 R, HPV15 R/HPV80 R, and HPV75 R/HPV76 R.

[b] F, forward; R, reverse.

RESULTS

Generation and characterization of an array primer extension assay for the detection of different HPV types. We selected 25 different cutaneous HPV types belonging to the *Betapapillomavirus* genus (HPV5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 49, 75, 76, 80, 92, 93, and 96). After alignment of the E7 DNA sequences from all selected HPV types, 25 pairs of primers specific for each HPV type were designed (Table 1). As a first step, by PCR, we tested the functionality of all primer pairs in single reactions using DNA of the corresponding HPV type as the template. Each pair of primers gave a PCR product of the expected size (data not shown). Next, we tested the efficiencies of HPV primers in a multiplex PCR. All primers were mixed, and multiplex PCR was performed using serial dilutions of DNA (from 1,000 to 0

TABLE 2. Sequences of the oligonucleotides spotted on the chip

HPV type	Oligonucleotide	Oligonucleotide sequence
5	1	5′-GGAAACGGAGGAGGAGCCTGACAACGAAAGGA-3′
	2	5′-CTTACAAAGTTATAGCTCCGTGCGGTTGCAGGAAC-3′
8	1	5′-CGCAACTGATTCGGGTATCAGGACCTTTCAAGAA-3′
	2	5′-GGAGCTAGACATCGAAAGAACTGTATTCAAAATTG-3′
9	1	5′-GACCTGCATTGTTACGAAGAATTGACAGAAGAACC-3′
	2	5′-TCTCACTCCCTACAAGATCGTAGCTGGCTGTGG-3′
12	1	5′-CAAACGAGCAGGAAACGGAGGAGGAGTCAGATA-3′
	2	5′-GTCAACCTTCGTATTTTTGTCAACGCAACTGATAC-3′
14	1	5′-CAGAATTTGCTCTTAGAACCTTCCAGAACCTGTTA-3′
	2	5′-GCTGCAAGGTTAAGCTTCGCATCTTTATAACTGC-3′
15	1	5′-TAAGTGAAGAAGAGACAGAGGAGGAGCCACGAT-3′
	2	5′-CTTACAAGATTGTAGTTCCGTGTTGCTTTTGTGAT-3′
17	1	5′-AGAAGAGACAGAGACAGAGGAGGAGCCTCGTCG-3′
	2	5′-CTCCGTGCTGCTTTTGTGGTTCTAAGCTACGGC-3′
19	1	5′-GAAACAGAGGAGGAGCCTGCTATTGAAAGATC-3′
	2	5′-CGCAATTTGGTATTAGAACCCTACAGGACATCC-3′
20	1	5′-AGAGAGGAGGAGCCTCAGATTGAAAGAGCCTCA-3′
	2	5′-GCGCTACAGAATTTGCTATTAGAAGCTTTCAACAA-3′
21	1	5′-CGCTACACAATTTGCTATTAGAACATTTCAGAATC-3′
	2	5′-ACAGAGGAGGAGCTACCAGAAAGGACCGCG-3′
22	1	5′-TTGACCTGCATTGCCACGAGGAGCTGCCTGAAC-3′
	2	5′-GCCTGAACTTCCAGAAGAGTTAGAAGAATCAGTGG-3′
23	1	5′-GAGGAGGAGCCTGAATACACTCCTTACAAGATCATCG-3′
	2	5′-TACGTGCTAGCCACAGATTTTGGAATTCGCTCG-3′
24	1	5′-CGATTATTTGTAGCAGCAACACAGTTTGGAATACG-3′
	2	5′-AGCTTACAAAATAATACTTTGTTGCGGCGGCGG-3′
25	1	5′-TTGTGAAAGCCACTGATTTTGGTATTAGAACAC-3′
	2	5′-AACAGAGGAGGAGCCTGCTATTGACAGAACTCCA-3′
36	1	5′-TTTTGTCCAAGCTACAGAATTTGGCATCAGAGCA-3′
	2	5′-TTCAAAGTCATTGCACCGTGTGGTTGCAGCCAC-3′
37	1	5′-CCGTGCTGCTTTTGTGGTTCTAAACTACGACTGA-3′
	2	5′-TGCAACGCCTATTGGAATTAGATCACAAGAAGAGC-3′
38	1	5′-GGAGCCAGCATACACCCCATACAAAATCATAGTTC-3′
	2	5′-CATACAAAATCATAGTTCTTTGTGGGGGTTGTGAAG-3′
47	1	5′-AGGCGGAGGAGGAGCTAGACATCGACAGAGTCG-3′
	2	5′-AACAAACCGTGGCATCAGGACATTTCAGGAAC-3′
49	1	5′-CTTATCCAAGGCATCCCTTACAAAGTTATTGCTAC-3′
	2	5′-GCCACTGACGCTGCTATTAGAAGTTTCCAAGAAC-3′
75	1	5′-TTCGTGTGTTTGTGCTTGCTACTGATCGGGCTA-3′
	2	5′-GCCAGCCCATTGACCTGCAATGCTACGAGGATT-3′
76	1	5′-CCAGCCCATTGACCTGCAATGCTACGAGAACT-3′
	2	5′-ACTGATCGGGCCATTCGAGAATTTCAATCTTTAC-3′
80	1	5′-AAGTGAAGAAGAGACAGAGGAGGAGCCACAACATA-3′
	2	5′-CATACAAGATTGTAGCTCCGTGTTGCTTCTGTGAA-3′
92	1	5′-GGAGCCTGCACCTCAAAGAATAGACTACAAGATAG-3′
	2	5′-TTTTGCATCGTGTACCCAATTTGGAATTAGAACTC-3′
93	1	5′-AGCCTGCAAGAACAGCTTTCAAGATTATTGTG-3′
	2	5′-TTCGAATATTTGTTGCTGCCACAGAATTTGGAG-3′
96	1	5′-TGTCAGAATCTACAGTAGCAGAGGTGGAGCC-3′
	2	5′-TGGAGGCTGTGATTCCCGGCTGAAAATCTACG-3′
β-Globin	1	5′-ATCACTTAGACCTCACCCTGTGGAGCCATACCC-3′
	2	5′-TCCTGAGGAGAAGTCTGCCGTTACTGCCC-3′

copies of viral genome) from different beta HPV types as the template. Representative experiments with HPV5, 12, 20, 24, and 36 DNAs are shown in Fig. 1A. A PCR product was obtained even when only 10 copies of viral genome for each HPV type were used as the template. Thus, the presence of a large number of primers in the PCR mix does not hamper the sensitivity of the assay. The addition of the β-globin primers to the multiplex PCR mix did not affect the detection efficiency (data not shown).

To determine the HPV types, after performing a multiplex PCR, we used an APEX assay. Two type-specific oligonucleotides covering two distinct regions of each E7 gene were synthesized for the 25 HPV types and were spotted in duplicate on the chip (Table 2 and Fig. 1B). After PCR, the amplified DNA fragments were hybridized to the chip and followed by an extension reaction in the presence of cyanine 5-ddUTP. To make the test simpler and applicable also for two-color scanners, we designed the chip oligonucleotides so that during the extension reaction the same dideoxynucleoside triphosphate (cyanine 5-ddUTP) would be incorporated in all cases. To test the specificity of the assay, we performed multiplex PCR using the individual HPV types as template. No false-positive HPV signals were detected by the APEX assay. In fact, in all cases, hybridization of the PCR product

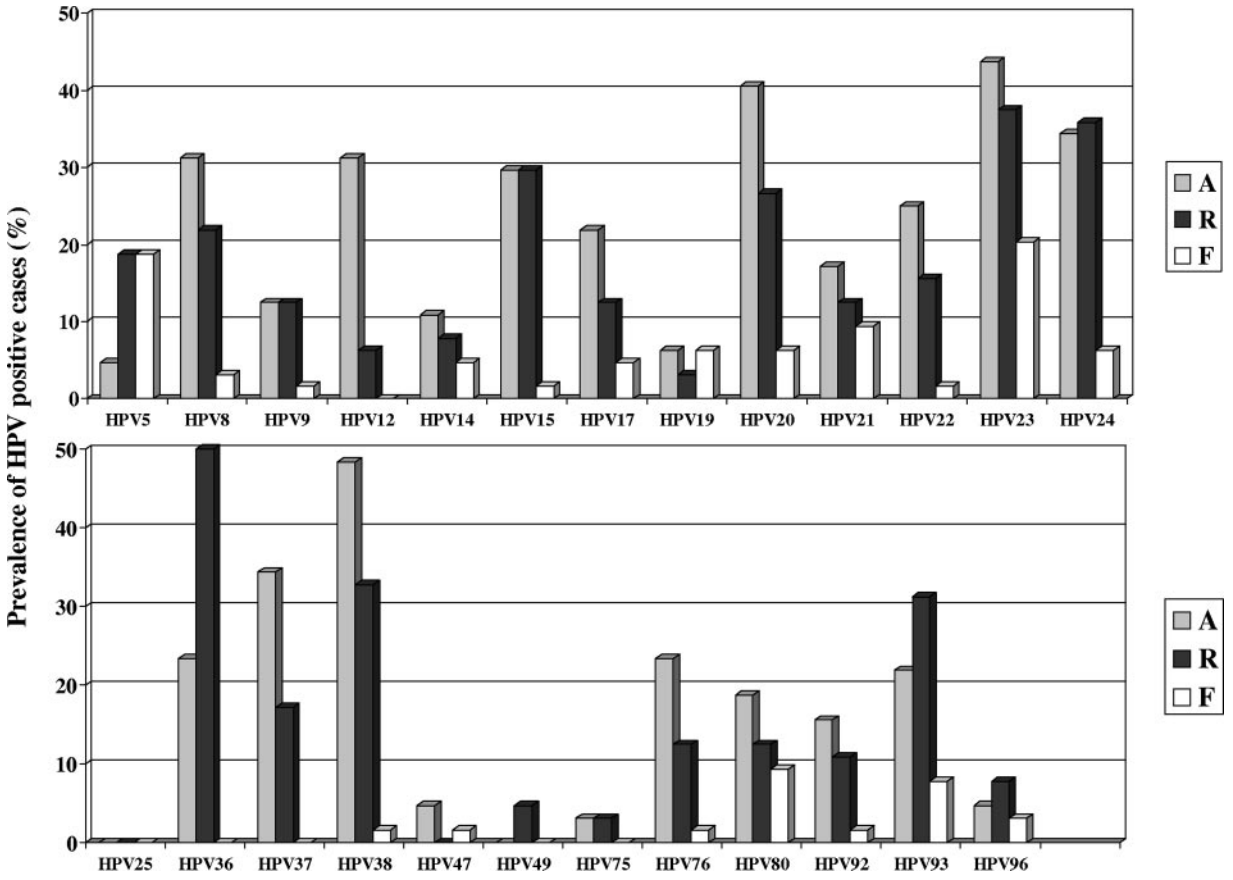


FIG. 2. Prevalence of 25 beta HPV types in human specimens. Prevalence is expressed as a percentage. Detection of the beta HPV DNA was determined in three different laboratories by three different HPV detection methods: (i) multiplex PCR/APEX assay (A); (ii) one-step PCR combined with a reverse hybridization assay (PM-PCR RHA method) (R); and (iii) FAP59/64 PCR method, followed by cloning and sequence of the PCR amplimers (F). For the HPV types detected by the FAP59/64 PCR method, specific HPV types and related family members were included (FA23.1, FA23.2, and FA23.3 related to HPV5; FA116 related to HPV9; FA84.2 related to HPV15; FA40 and FA140 related to HPV17; FA14 related to HPV19; FA130 related to HPV20; FA25 related to HPV21; FA16.1, FA118, FA123, and FAIMVS13.2 related to HPV23; FA18, FA119, and FA122 related to HPV24; FA125 related to HPV38; FA26 related to HPV47; FA5, FA75, FA114, and FAIMVS14 related to HPV80; FAIMVS6.1, FAIMVS6.2, and FAIMVS6.5 related to HPV93; FA7 and FA74 related to HPV96).

on the chip gave a positive signal for each HPV type (data not shown).

Figure 1C shows a representative HPV typing experiment in which a suspension of cells from a basal cell carcinoma swab was used as the PCR template (see Materials and Methods for more details). Seven different HPV types were clearly detected (HPV8, 15, 22, 24, 37, 38, and 76). In addition, DNA quality was monitored using β-globin as the internal control (Fig. 1C).

Together, these data indicate that the combination of multiplex PCR with the APEX microarray method is both specific and sensitive enough to detect the beta HPV types.

Intermethod comparison. To further validate the multiplex PCR/APEX assay, we blindly analyzed 64 samples from NMSC and other skin lesions, such as keratoacanthomas, actinic keratoses, seborrheic keratoses, and other benign lesions (see Materials and Methods for more details). The skin specimens were analyzed in three different laboratories by three different HPV detection methods: (i) multiplex PCR/APEX assay; (ii) one-step PCR combined with a reverse hybridization assay (PM-PCR RHA method); and (iii) FAP59/64 PCR method, followed by cloning and sequence of the PCR amplimers.

Although the FAP59/64 PCR method allowed the identification of several unknown HPV types, its sensitivity for the 25 known beta HPV types was clearly lower than those of the PM-PCR RHA and multiplex PCR-APEX methods (Fig. 2). The last two methods detected similar percentages of beta HPV-positive samples (92.2% with multiplex PCR/APEX assay versus 90.6% with PM-PCR RHA), and the concordance between the two assays in identifying HPV-negative ($n = 3$) or HPV-positive ($n = 56$) samples was over 92% (Table 3). The three beta HPV-negative samples were one basal cell carcinoma, one keratoacanthoma, and one seborrheic keratosis. In agreement with previous studies, the majority of skin specimens contained multiple HPV infections. However, our method detected a higher number of multiple infections than the PM-PCR RHA assay did (Table 4). The multiplex PCR/APEX showed the presence of 325 HPV infections in the 64 skin specimens, while 271 were detected by the PM-PCR RHA assay (Table 4).

To confirm the specificity of our assay and to exclude the possibility of false-positive results, the presence of specific HPV types was also determined by PCR followed by direct

TABLE 3. Agreement between two different HPV detection methods (multiplex PCR/APEX and PM-PCR RHA) of 25 beta HPV types using skin specimens[a]

Beta HPV type	No. (%) of HPV-positive specimens by:			Kappa (95% CI)
	Multiplex PCR/APEX method only[b]	PM-PCR-RHA method only	Both methods	
5	0 (0.0)	9 (14.1)	3 (4.7)	35 (5–65)
8	8 (12.5)	2 (3.1)	12 (18.8)	60 (39–82)
9	6 (9.4)	6 (9.4)	2 (3.1)	14 (0–45)
12	16 (25.0)	0 (0.0)	4 (6.3)	26 (4–47)
14	2 (3.1)	0 (0.0)	5 (7.8)	82 (57–100)
15	4 (6.3)	4 (6.3)	15 (23.4)	70 (51–89)
17	9 (14.1)	3 (4.7)	5 (7.8)	35 (7–63)
19	4 (6.3)	2 (3.1)	0 (0.0)	0 (0–0)
20	11 (17.2)	2 (3.1)	15 (23.4)	55 (35–76)
21	3 (4.7)	0 (0.0)	8 (12.5)	82 (61–100)
22	7 (10.9)	1 (1.6)	9 (14.1)	62 (39–85)
23	11 (17.2)	7 (10.9)	17 (26.6)	42 (20–64)
24	5 (7.8)	6 (9.4)	17 (26.6)	62 (42–82)
25	0 (0.0)	0 (0.0)	0 (0.0)	0
36	2 (3.1)	19 (29.7)	13 (20.3)	34 (15–54)
37	12 (18.8)	1 (1.6)	10 (15.6)	49 (27–71)
38	12 (18.8)	2 (3.1)	19 (29.7)	56 (36–75)
47	3 (4.7)	0 (0.0)	0 (0.0)	
49	0 (0.0)	3 (4.7)	0 (0.0)	
75	1 (1.6)	1 (1.6)	1 (1.9)	48 (0–100)
76	8 (12.5)	1 (1.6)	7 (10.9)	53 (27–79)
80	4 (6.3)	0 (0.0)	8 (12.5)	76 (55–98)
92	5 (7.8)	2 (3.1)	5 (7.8)	53 (22–83)
93	3 (4.7)	9 (14.1)	11 (17.2)	52 (29–75)
96	1 (1.6)	3 (4.7)	2 (3.1)	47 (3–91)
Overall HPV positive	3 (4.7)	2 (3.1)	56 (87.5)	50 (13–88)

[a] Three specimens were HPV negative by both methods (4.7%).
[b] This analysis was performed using the 5-1 primers.

TABLE 4. Distribution of concurrent multiple HPV infections as detected by the multiplex PCR/APEX and PM-PCR RHA method

No. of concurrent HPV types	APEX assay[a]		RHA	
	No. of subjects (%)[b]	No. of infections	No. of subjects (%)	No. of infections
0	5 (7.8)	0	6 (9.4)	0
1	3 (4.7)	3	5 (7.8)	5
2 or 3	13 (20.3)	35	15 (23.4)	37
4 or 5	19 (29.7)	83	20 (31.3)	91
6 or 7	8 (12.5)	52	11 (17.2)	70
8 or 9	9 (14.1)	77	5 (7.8)	44
≥10	7 (10.9)	75	2 (3.1)	24
Total	64 (100.0)	325	64 (100.0)	271

[a] This analysis was performed using the 5-1 primers.
[b] The denominator is the total number of subjects, i.e., 64.

DNA sequencing in 20 samples. After the PCR, we performed DNA sequencing using HPV type-specific primers. In all cases, DNA sequencing confirmed the data obtained with the multiplex PCR/APEX assay (data not shown). Most importantly, DNA sequencing confirmed the presence of nine additional HPV types in six cases that were detected by the multiplex PCR/APEX assay but not by the PM-PCR RHA method (data not shown).

As shown in Fig. 2 and Table 3, our method detected a larger number of the following beta HPV types: HPV8, 12, 17, 20, 22, 37, 38, and 76. In contrast, the PM-PCR RHA method clearly detected more HPV5-, 36-, and 93-positive infections than the multiplex PCR/APEX assay did (12 versus 3, 32 versus 15, and 20 versus 14, respectively). The low sensitivity of our assay for HPV5 detection was most likely due to the fact that the reverse (R) PCR primer contains an A/T degeneration for the presence of a variant in HPV5 E7 (Table 1, primers 5-1).

Next, we determined the reproducibility of our assay in comparison to the PM-PCR RHA methods. Ten randomly selected specimens were examined in a blind manner by multiplex PCR/APEX or PM-PCR RHA. To overcome the problem with the detection of HPV5, we designed a new pair of PCR primers (Table 1, primers 5-2) that amplified a different fragment of the HPV5 E7. The multiplex PCR/APEX assay with the new HPV5 primers showed a similar sensitivity to the PM-PCR RHA method in detecting HPV5-positive infections in 10 of 64 skin specimens (Table 5). In Table 5, we have identified three HPV5 infections in triplicate that gave negative results when the first pairs of HPV5 E7 primers were used.

Table 5 also shows that the majority of the HPV infections were detected in triplicate by both assays and that the percentage of positive agreement was similar between the two methods (70.1% and 95% confidence interval [95% CI] of 55.9 to 83.0 for multiplex PCR/APEX; 75.0% and 95% CI of 57.8 to 87.9 for the PM-PCR RHA.

DISCUSSION

Recent biological and epidemiological studies support the involvement of the beta HPV types in the development of NMSC (16). However, due to the high heterogeneity and ubiquity of these viruses, it is not clear yet whether specific beta HPV types are more prevalent than others in malignant skin lesions. Although the majority of epidemiological studies have shown that the beta HPV types are present in a large proportion of NMSC (up to 90%), there is no consensus on the spectrum of HPV types detected in the different investigations. The apparent discrepancy among the different studies is probably due to the fact that a wide variety of PCR primers are used in the HPV DNA detection methods. The majority of the assays use different sets of degenerate and/or consensus primers that have different sensitivities in detecting the different beta HPV types. Since multiple infections are extremely frequent in the skin, it is conceivable that, due to the primer competition, the use of degenerate and/or consensus primers may result in underdetection of several HPV types.

In the present study, we describe a multiplex PCR-based method for the detection of 25 beta HPV types. This technique offers the advantages of the multiplex PCR methods, i.e., high sensitivity and the possibility to perform multiple amplifications in a single reaction mixture. HPV typing was achieved with an APEX assay, which offers the specificity of Sanger dideoxy sequencing with the high throughput potential of the microarray (12, 13, 15, 21). The multiplex PCR/APEX method was seen to be highly specific, since two oligonucleotides corresponding to different regions of E7 PCR product were spot-

TABLE 5. Results of triplicate MP-APEX and PM-RHA performed on 10 samples chosen randomly among 64 samples

Sample no.	Assay no.	HPV type[a] detected by:	
		Multiplex APEX assay[b]	PM-RHA
1	1	**HPV5**, **8**, **20**	**HPV8**, 20
	2	**HPV5**, **8**, **20**	HPV5, **8**
	3	**HPV5**, **8**, **20**	**HPV8**, 20
2	1	**HPV20**, **23**, **38**, **80**, **92**	**HPV17**, **20**, **23**, **76**, **80**, **92**, **93**
	2	**HPV20**, **23**, **38**, **80**, **92**	**HPV17**, **20**, **23**, 38, **76**, **80**, **92**, **93**
	3	**HPV20**, **23**, **38**, **80**, **92**	**HPV17**, **20**, **23**, 38, **76**, **80**, **92**, **93**
3	1	**HPV22**, **38**	**HPV38**
	2	**HPV22**, **38**	**HPV38**
	3	**HPV22**, **38**	**HPV38**
4	1	**HPV5**, 8, **12**, 17, **20**, **23**, 80	Negative
	2	**HPV5**, **12**, 17, **20**, **23**, 80	Negative
	3	**HPV5**, **12**, **20**, **23**	Negative
5	1	HPV8, **9**, **15**, **17**, **20**, **22**, **23**, **24**, **36**, **37**, **38**, **47**, **75**, **80**, 92	**HPV8**, **9**, **15**, **20**, **22**, **23**, **24**, **36**, **37**, **38**, **49**, **75**, **76**, **80**, **93**, **96**
	2	HPV8, **9**, 12, **15**, **17**, **20**, **22**, **23**, **24**, **36**, **37**, **38**, **47**, **75**, **80**, 92	**HPV8**, **9**, **15**, 17, **20**, **22**, **23**, **24**, **36**, **37**, **38**, **49**, **75**, **76**, **80**, **93**, **96**
	3	**HPV9**, 12, **15**, **17**, **20**, **22**, **23**, **24**, **36**, **37**, **38**, **47**, **75**, **80**	**HPV8**, **9**, **15**, 17, **20**, **22**, **23**, **36**, **37**, **38**, **49**, **75**, **76**, **80**, **93**, **96**
6	1	**HPV5**, **15**, 19, 20, 21, **22**, **23**, **38**, **80**, **92**	**HPV15**, **23**, **38**, 92
	2	**HPV5**, **15**, 20, 21, **22**, **23**, **38**, **80**, **92**	**HPV15**, **23**, **38**
	3	**HPV5**, **15**, 19, **22**, **23**, **38**, **80**, **92**	**HPV15**, 19, **23**, **38**
7	1	Negative	HPV36
	2	Negative	HPV+[c]
	3	Negative	Negative
8	1	HPV36, 93	HPV36, **93**
	2	HPV36	**HPV93**
	3	HPV93	**HPV93**
9	1	Negative	HPV+
	2	HPV36	HPV+
	3	HPV8	HPV+
10	1	Negative	HPV+
	2	Negative	HPV+
	3	HPV5	Negative

[a] The types detected in triplicate are shown in bold type.
[b] This analysis was performed using the 5-2 and 36-2 F primers.
[c] HPV+ indicates that a HPV was found with a universal probe but no signal was detected with the type-specific probes.

ted on the chip for each HPV type (13). In fact, when the assay was performed using cloned HPV genomes, no cross-reaction was observed among the different HPV types. In addition, the multiplex PCR was shown to be highly sensitive, being able to give a positive signal even when only 10 copies of viral genome were used as the template.

Natural variations within the HPV genome may have an impact on the sensitivity of HPV detection methods. In fact, the first version of our assay was clearly less efficient than the PM-PCR RHA method in identifying HPV5 infections. Due to the general features of the multiplex PCR-APEX assay, namely, high versatility and flexibility, the sensitivity for HPV5 was easily increased by modification of the PCR primers (Table 5; T. Gheit et al., unpublished data). Another advantage of our novel assay is the possibility to amplify, together with HPV DNA, a region of the β-globin gene, which is an important criterion for evaluating the quality of DNA used in the analysis.

We validated the multiplex PCR/APEX method by analyzing skin swabs and comparing its performance with two previously described HPV detection methods, i.e., FAP59/64 PCR (10) and PM-PCR RHA (8). The FAP59/64 PCR assay detected several unknown cutaneous HPV types but showed very low agreement with the other methods in detecting the 25 beta HPV types. In fact, using the FAP59/64 PCR method, the identification of HPV type is achieved by cloning and sequencing of the PCR products. It is highly likely that with this procedure many HPV types are missed, especially in multiple infections. However, the sensitivity of the FAP59/64 PCR has recently been improved by using a nested PCR approach (11). The multiplex PCR/APEX assay showed over 90% agreement with the PM-PCR RHA method. However, the novel assay clearly detected a higher number of infections than the PM-PCR RHA did in the 64 skin specimens (325 versus 271, respectively). The higher sensitivity of our assay is most likely due to the use of type-specific PCR primers. In these conditions, the majority of HPV types present in the same specimens will not compete for the same primer set, which is not the case when degenerate and/or consensus primers are used. One limitation of the multiplex PCR/APEX assay could be the use of PCR primers that amplify DNA fragments of approximately 200 to 300 bp. This aspect becomes very important when studies have to be conducted on poor quality DNA, since the amplification of relatively large PCR products may be hampered by DNA fragmentation. However, we have preliminary data showing that our assay is able to efficiently detect beta HPV types when DNA is extracted from paraffin-embedded tissues, skin swabs, and plucked eyebrow hairs (Gheit et al., unpublished).

In conclusion, this study describes a novel assay for detection of 25 beta HPV types with high sensitivity and specificity, which is applicable for epidemiological studies aimed at determining

the distribution of various beta HPV types in skin specimens and characterizing their role in skin diseases.

ACKNOWLEDGMENTS

We are grateful to all members of our laboratory for their cooperation and Uzma Hasan for critical reading of the manuscript.

The work was partially supported by a grant from the Association pour la Recherche sur le Cancer to B.S.S. and a grant from the Association for International Cancer Research to M.T.

REFERENCES

1. **Alotaibi, L., N. Provost, S. Gagnon, E. L. Franco, and F. Coutlee.** 2006. Diversity of cutaneous human papillomavirus types in individuals with and without skin lesion. J. Clin. Virol. **36:**133–140.
2. **Altman, D. G.** 1991. Practical statistics for medical research. Chapman and Hall, London, United Kingdom.
3. **Antonsson, A., O. Forslund, H. Ekberg, G. Sterner, and B. G. Hansson.** 2000. The ubiquity and impressive genomic diversity of human skin papillomaviruses suggest a commensalic nature of these viruses. J. Virol. **74:**11636–11641.
4. **Auffray, C., C. Mundy, and A. Metspalu.** 2000. DNA arrays: methods and applications: report on HUGO Meeting, Tartu, Estonia, 23–26 May, 1999. Eur. J. Hum. Genet. **8:**236–238.
5. **Berkhout, R. J., L. M. Tieben, H. L. Smits, J. N. Bavinck, B. J. Vermeer, and J. ter Schegget.** 1995. Nested PCR approach for detection and typing of epidermodysplasia verruciformis-associated human papillomavirus types in cutaneous cancers from renal transplant recipients. J. Clin. Microbiol. **33:**690–695.
6. **Bouvard, V., A. S. Gabet, R. Accardi, S. B. Sylla, and M. Tommasino.** 2006. The cutaneous human papillomaviruses and non-melanoma-skin cancer. Caister Academic Press, Norfolk, United Kingdom.
7. **Brink, A. A. T. P., B. Lloveras, I. Nindl, D. A. Heideman, D. Kramer, R. Pol, M. J. Fuente, C. J. L. M. Meijer, and P. J. F. Snijders.** 2005. Development of a general-primer-PCR-reverse-line-blotting system for detection of beta and gamma cutaneous human papillomaviruses. J. Clin. Microbiol. **43:**5581–5587.
8. **de Koning, M., W. Quint, L. Struijk, B. Kleter, P. Wanningen, L. J. van Doorn, S. J. Weissenborn, M. Feltkamp, and J. ter Schegget.** 2006. Evaluation of a novel highly sensitive, broad-spectrum PCR-reverse hybridization assay for detection and identification of beta-papillomavirus DNA. J. Clin. Microbiol. **44:**1792–1800.
9. **de Villiers, E. M., C. Fauquet, T. R. Broker, H. U. Bernard, and H. zur Hausen.** 2004. Classification of papillomaviruses. Virology **324:**17–27.
10. **Forslund, O., A. Antonsson, P. Nordin, B. Stenquist, and B. G. Hansson.** 1999. A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin. J. Gen. Virol. **80:**2437–2443.
11. **Forslund, O., H. Ly, and G. Higgins.** 2003. Improved detection of cutaneous human papillomavirus DNA by single tube nested 'hanging droplet' PCR. J. Virol. Methods **110:**129–136.
12. **Gemignani, F., S. Landi, A. Chabrier, A. Smet, I. Zehbe, F. Canzian, and M. Tommasino.** 2004. Generation of a DNA microarray for determination of E6 natural variants of human papillomavirus type 16. J. Virol. Methods **119:**95–102.
13. **Gheit, T., S. Landi, F. Gemignani, P. J. Snijders, S. Vaccarella, S. Franceschi, F. Canzian, and M. Tommasino.** 2006. Development of a sensitive and specific assay combining multiplex PCR and DNA microarray primer extension to detect high-risk mucosal human papillomavirus types. J. Clin. Microbiol. **44:**2025–2031.
14. **Guo, Z., R. A. Guilfoyle, A. J. Thiel, R. Wang, and L. M. Smith.** 1994. Direct fluorescence analysis of genetic polymorphisms by hybridization with oligonucleotide arrays on glass supports. Nucleic Acids Res. **22:**5456–5465.
15. **Kurg, A., N. Tonisson, I. Georgiou, J. Shumaker, J. Tollett, and A. Metspalu.** 2000. Arrayed primer extension: solid-phase four-color DNA resequencing and mutation detection technology. Genet. Test. **4:**1–7.
16. **Pfister, H.** 2003. Chapter 8: human papillomavirus and skin cancer. J. Natl. Cancer Inst. Monogr. **31:**52–56.
17. **Pfister, H., P. G. Fuchs, S. Majewski, S. Jablonska, I. Pniewska, and M. Malejczyk.** 2003. High prevalence of epidermodysplasia verruciformis-associated human papillomavirus DNA in actinic keratoses of the immunocompetent population. Arch. Dermatol. Res. **295:**273–279.
18. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
19. **Shamanin, V., H. Delius, and E. M. de Villiers.** 1994. Development of a broad spectrum PCR assay for papillomaviruses and its application in screening lung cancer biopsies. J. Gen. Virol. **75:**1149–1156.
20. **Shamanin, V., M. Glover, C. Rausch, C. Proby, I. M. Leigh, H. zur Hausen, and E. M. de Villiers.** 1994. Specific types of human papillomavirus found in benign proliferations and carcinomas of the skin in immunosuppressed patients. Cancer Res. **54:**4610–4613.
21. **Shumaker, J. M., A. Metspalu, and C. T. Caskey.** 1996. Mutation detection by solid phase primer extension. Hum. Mutat. **7:**346–354.
22. **Surentheran, T., C. A. Harwood, P. J. Spink, A. L. Sinclair, I. M. Leigh, C. M. Proby, J. M. McGregor, and J. Breuer.** 1998. Detection and typing of human papillomaviruses in mucosal and cutaneous biopsies from immunosuppressed and immunocompetent patients and patients with epidermodysplasia verruciformis: a unified diagnostic approach. J. Clin. Pathol. **51:**606–610.
23. **zur Hausen, H.** 2002. Papillomaviruses and cancer: from basic studies to clinical application. Nat. Rev. Cancer **2:**342–350.