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Betapapillomavirus infections : natural history and disease association

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CHAPTER 2

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Evaluation of a Novel Highly Sensitive, Broad-Spectrum PCR-Reverse Hybridization Assay for Detection and Identification of Beta-Papillomavirus DNA

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Human papillomavirus can be detected by amplification of viral DNA. A novel one-step PCR (PM-PCR) was evaluated for amplification of a 117-bp fragment from the E1 region. It permitted ultrasensitive detection of all 25 known human papillomavirus genotypes from the beta-papillomavirus genus. The intra- and intertypic sequence variations of the 77-bp interprimer region were studied. Genotype-specific probes as well as general probes were selected for the 25 established beta-papillomavirus types, and a reverse hybridization assay (RHA) was developed (PM-PCR RHA method). The analytical sensitivity of the PM-PCR RHA method was 10 to 100 viral genomes. The one-step PM-PCR turned out to be more sensitive than the previously described nested MaHa-PCR for beta-papillomavirus detection. The PM-PCR RHA method was able to detect and identify beta-papillomavirus types in frozen patient material as well as in poorly amplifiable material such as formalin-fixed, paraffin-embedded skin biopsy specimens. Inter- and intralaboratory variability experiments showed that the reproducibility of the assay was very high. In conclusion, the one-step PM-PCR together with the RHA allows extremely sensitive, specific, and reproducible detection of beta-papillomavirus DNA as well as reliable identification of beta-papillomavirus genotypes in both fresh and paraffin-embedded patient material.

Papillomaviruses (PV) constitute a group of viruses associated with benign and malignant lesions of cutaneous and mucosal epithelia. So far, more than 100 different PV genotypes have been identified, of which approximately 48 types have been detected in human cutaneous lesions (12). These include the beta-papillomavirus (beta-PV) genus comprising the human papillomavirus (HPV) types 5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 49, 75, 76, 80, and 93 and candidate types 92 (cand92) and cand96. Based on partial sequences, however, probably more than 35 new types have to be added to the 25 known beta-PV types (19). Originally, types of the beta genus have been found in skin lesions from patients with the rare hereditary disease epidermodysplasia verruciformis. These patients develop flat cutaneous warts and macular lesions. They arise early in life and have a high chance to progress into squamous cell carcinoma (SCC) on sun-exposed sites. In these SCCs, mostly HPV types 5 and 8 have been detected, suggesting that these types are high-risk HPV types (19).

DNA from beta-PV types was identified mainly by nested PCR in 30 to 50% of SCCs in immunocompetent patients and in up to 90% of the SCCs in immunosuppressed patients, e.g., renal transplant recipients (19). The high prevalence of beta-PV types in these SCCs and their precursor lesions (solar keratoses) suggests an involvement in the carcinogenesis. Recent epidemiological case control studies have further corroborated this hypothesis by showing that the presence of beta-PV DNA in eyebrow hairs was associated with a history of cutaneous solar keratoses (7) and cutaneous SCC (23).

Little is known about the biological properties of the beta-PV types and the putative mechanism of beta-PV-related carcinogenesis. At present, only the biological properties of the beta-PV types 20 and 38 have been studied in some detail in primary human keratinocytes. In contrast to HPV type 20 (HPV20), HPV38 E7 is able to inactivate the tumor suppressor pRb and induces loss of G_1/S transition control. Furthermore, HPV38 E6 and E7 are sufficient to deregulate the cell cycle and senescence programs in primary human keratinocytes (9). The carcinogenic potential of the HPV type 8 early region was recently shown in a transgenic mouse model (21). The available data suggest that HPV8 and possibly also other beta-PV types like HPV5 and HPV38 are high risk and analogous to high-risk genital HPV types.

To study an association between one or more specific beta-PV types and SCC development, large epidemiologic case control and cohort studies are needed. These studies require the accurate detection and genotyping of HPV DNA in a large number of samples, often containing multiple beta-PV types.

In the last two decades, several broad-spectrum PCR methods to detect skin HPV types have been described, such as the following: CPI/IIs (25), FAP59/64 (13), F/G (3), modified F/G (MaHa) (5), and HVP-PCR (22). Several PCR approaches were also described by Harwood and coworkers (15).

Broad-spectrum PCR methods combined with either cloning and sequencing or direct sequencing of the amplimers and type-specific PCRs are widely accepted for beta-PV genotyping. These methods are clearly too laborious for large epidemiological studies and will lead to an underestimation of the number of types present (20, 26). Earlier experiences with the

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established SPF10-LiPA (16) system for diagnosis of anogenital HPV genotypes show that a broad-spectrum consensus PCR combined with a reverse hybridization assay is well suited for the identification of HPV types in large studies.

In the present study, we evaluate a newly developed broad-spectrum PCR (PM-PCR) in combination with a reverse hybridization system (RHA) for rapid genotyping of HPV types belonging to the beta genus. The PM-PCR RHA method is compared with the already established MaHa broad-spectrum PCR, and the application of this method in different clinical materials like eyebrow hairs and paraffin-embedded skin biopsy specimens is studied.

MATERIALS AND METHODS

Clinical materials. Eyebrow hair samples were randomly selected from three studies. The first is the Leiden Skin Cancer Study (10), a hospital-based, case control study of 1,126 subjects, including patients with squamous cell carcinoma, basal cell carcinoma, malignant melanoma, and control subjects. The second study (unpublished) includes samples from 23 healthy individuals from The Netherlands. The third is an ongoing case control study of renal transplant recipients, including 190 patients and controls, assessing the association between beta-PV and SCC (kindly provided by S. Euvrard).

Hair sampling was performed by taking 8 to 10 eyebrow hairs from every subject with a sterile pair of tweezers and gloves (6). The samples were kept frozen in Eppendorf tubes with screw caps with external threading to prevent loss of material and contamination due to hairs getting stuck in the threading.

Isolated DNA from paraffin-embedded shaved biopsy specimens, approximately 3 mm in diameter, was obtained from R. B. Harris (Arizona Cancer Center, Tucson, Ariz.) and M. Tommasino (International Agency for Research on Cancer, World Health Organization, Lyon, France). The biopsy specimens were taken with a scalpel from healthy forearm and underarm skin of SCC patients and controls. Prior testing using the RLB technique recently described (8) showed that 9 of 20 samples were beta-PV positive (C. M. Nielson and R. B. Harris, Arizona Cancer Center, University of Arizona, personal communication).

DNA isolation. DNA isolation from eyebrow hairs was carried out with the guanidine-thiocyanate-diatom method described by Boom et al. (4) or with the QIAamp DNA mini kit (QIAGEN GmbH, Hilden, Germany) or the High-Pure PCR template purification (Roche Diagnostics, Alameda, CA). Briefly, for the Boom method, clinical materials were treated with 400 μl of the chaotropic agent guanidine-thiocyanate. Two-thirds of the volume of this lysed material was stored at −70°C while the rest was further purified by binding to silica particles, followed by several washing steps. Finally, DNA was eluted from the silica in 100 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) during 10 min of incubation at 56°C and stored at −20°C. Briefly, for the QIAamp DNA mini kit isolation, clinical materials were pretreated overnight with the proteinase K solution according to the manufacturer's instructions. After lysis with 200 μl buffer AL, half of the volume was stored at −70°C while the other half was further processed according to the manufacturer's instructions. DNA was eluted in 100 μl of elution buffer AE and stored at −20°C. DNA from the paraffin-embedded shaved biopsy specimens was isolated with the High-Pure PCR template purification kit. The DNA was eluted in 100 μl of elution buffer, provided in the kit.

Plasmids. Plasmids containing partial or complete HPV genomic DNA were kindly provided by R. S. Ostrow, Minnesota (HPV genotype 5), G. Orth, Paris, France (HPV genotypes 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, and 49), E.-M. de Villiers, Heidelberg, Germany (HPV genotypes 8, 37, 38, 75, 76, and 80), M. Ishibashi, Nagoya, Japan (HPV genotype 47), T. Matsukura, Tokyo, Japan (HPV genotypes 65 and 67), and O. Forslund, Malmö, Sweden (HPV genotype 93 and candidate genotypes 92 and 96).

HPV plasmid DNA concentrations were measured with Hoechst 33258. Briefly, 0.5 mg/ml Hoechst 33258 stock solution was prepared and subsequently diluted 1:2,500 in 1× TNE buffer (10 mM Tris base, 0.2 M NaCl, 1 mM EDTA, pH 7.4) to give a final Hoechst 33258 assay solution concentration of 200 ng/ml. The DNA standard solutions were prepared by serial dilution of pGEMZ3f(+) (0.2 mg/ml) in Hoechst 33258 assay solution, ranging from 10 to 500 ng/ml. To estimate the concentration and purity of the plasmid HPV DNA, the absorbance of the DNA solution was measured at 260, 280, and 320 nm using UV spectrometry. Subsequently, this plasmid HPV DNA was diluted in Hoechst 33258 assay solution to give a final estimated concentration ranging from 50 to 400 ng/ml. Plasmid HPV DNA solutions were stored in the dark at room temperature and prepared less than 1 h prior to use. The Hoechst 33258 fluorescence was measured with a spectrofluorometer at 365 nm and 460 nm at room temperature. Finally, the fluorescence sample readings were performed in duplex reaction mixtures and were calculated as the means of 20 fluorescence measurements. The unknown plasmid HPV DNA concentration was calculated utilizing the standard curve.

Tenfold serial dilution of plasmid clones of HPV types 5, 8, 9, 15, 17, 19, 23, 24, 36, 38, 49, 93, and cand96 were made for analytical sensitivity testing. The dilution series ranged from 10,000 to 0.1 copies of plasmid in a background of 5 ng human genomic DNA/μl. This background human DNA is equivalent to approximately 10,000 cells per 10 μl PCR.

The PM-PCR RHA method. The PM-PCR RHA method [Skin (beta) HPV prototype research assay; Diassay BV, The Netherlands] comprises the PM-PCR generating a biotinylated amplimer of 117 bp from the E1 region and an RHA able to simultaneously identify 25 beta-PV genotypes.

Within the E1 gene of beta-PV genotypes, two relatively well-conserved regions suitable for the design of a broad-spectrum PCR were found (regions A and D) (Fig. 1). The primer selection was aimed at minimizing the number of mismatches with each targeted genotype and at minimizing the required number of primers, resulting in a primer set consisting of nine nondegenerated primers (two forward and seven reverse) without inosines.

The PCR was carried out with all precautions to avoid contamination as described by the manufacturer.

Briefly, PM-PCR was performed in a final reaction volume of 50 μl, containing 10 μl of the isolated DNA, 2.5 mM $MgCl_2$, 1× GeneAmp PCR buffer II, 0.2 mM concentrations of deoxynucleoside triphosphates, 1.5 U Ampli*Taq* Gold DNA polymerase, and 10 μl of the PM primer mix. The PCR was performed by a 9-min preheating step at 94°C, followed by 35 cycles of amplification comprising 30 s at 94°C, 45 s at 52°C, and 45 s at 72°C. A final elongation step at 72°C of 5 min ends the PCR. The MaHa PCR was carried out as described previously (5, 11).

The RHA (24) allows the simultaneous identification of multiple HPV genotypes in a single hybridization step. Sequence alignments from the PM amplimers (Fig. 1) showed a relatively variable region (region C) that allowed the deduction of 27 genotyping probes. Twenty-three of these probes are genotype specific, while the four other probes are used in pattern recognition of genotypes. Since the 25 beta-PV genotypes often differ by only a few nucleotides in the 77-bp interprimer sequences (Fig. 1), well-controlled hybridization conditions and probe selection is needed. A relatively conserved region (region B) permitted the design of additional general probes for broad-spectrum beta-PV detection. These general probes were developed for the detection of at least the established beta-PV types, with the exception of types 38, 92, and 96. The additional probes for broad-spectrum detection of beta-PV genotypes were mixed and applied to the strip as a single probe line. The top line (conjugate control) contains a positive control of biotinylated DNA. The outline of the RHA strip and representative examples of the beta-PV RHA are depicted in Fig. 2. In most cases, interpretation of the test result is directly linked to the probe name of the HPV type (e.g., a purple color on probe line HPV5 indicates the presence of HPV5) (Fig. 2). However, there are some exceptions for genotypes yielding a more complex hybridization pattern.

For HPV8, two probes are present for its identification (probe HPV8 I and probe HPV8 II). HPV8 can be identified by two reaction patterns: (i) a positive reaction of probes HPV8 I and II or (ii) due to a higher sensitivity of probe HPV8 II, a single reaction can be expected with this probe in samples with a low HPV8 viral load. However, it should be noted that HPV47 also reacts with probe HPV8 II. Thus, only when HPV47 is absent (no reactivity on probe HPV47) can HPV8 be identified by a single reaction for probe HPV8 II alone.

Two probes are used for the identification of HPV type 21. Both probes cHPV21 and HPV21 should be positive for identification of HPV21. Probe cHPV21 can also react with amplimers of HPV types 20 and 22.

In summary, HPV genotypes 5, 9, 12, 14, 15, 17, 19, 23, 24, 25, 36, 37, 38, 49, 75, 76, 80, cand92, 93, and cand96 are recognized by hybridization to a single probe line, whereas HPV types 8 and 21 yield a specific hybridization pattern on the RHA. HPV genotypes 20, 22, and 47 are identified by a single probe line as well as by a specific reaction pattern.

The RHA was performed according to the kit insert. Briefly, 10 μl of the biotin-labeled amplimer was mixed with 10 μl of denaturation solution and 10 μl of 3B buffer in a plastic trough containing the beta-PV strip. The mix was incubated for 5 min at room temperature. Two milliliters of prewarmed (37°C) hybridization buffer (3× SSC [1× SSC is 15 mM Na citrate and 150 mM NaCl], 0.1% sodium dodecyl sulfate) was added and incubated at 50 ± 0.5°C for 1 h. All incubations and washing steps were performed automatically in an Auto-LiPA. The strips were washed twice for 30 s and once for 30 min at 50°C with 2 ml of

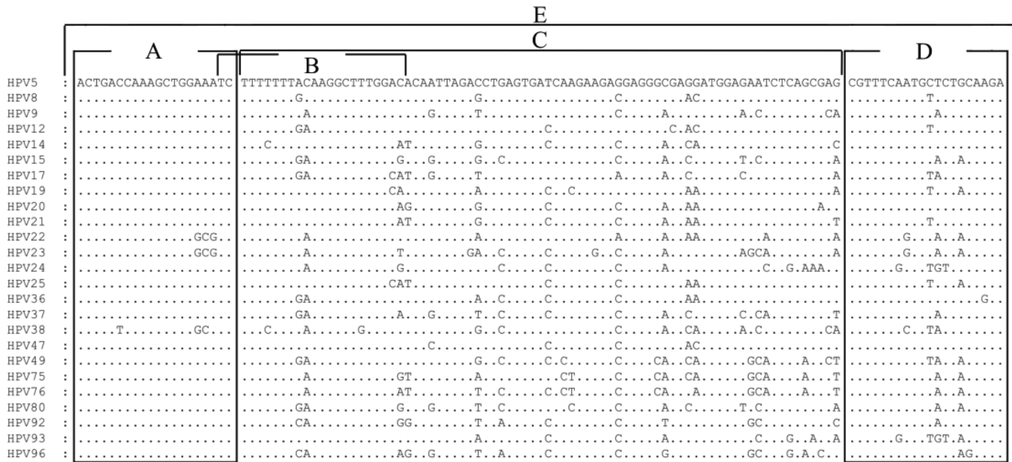

FIG. 1. Nucleotide sequence alignment of the target region for the PM-PCR for 25 beta-PV types. The complete 117-bp product is designated region E and is located between nucleotides 2644 and 2760. The sequence numbering is relative to the HPV5 sequence. Target regions A and D for the forward and reverse primers, respectively, are shown by boxes. The 77-bp area between the primers is designated region C. Region B is the target for the universal probes. The nucleotides identical to the top sequence are indicated by dots.

hybridization solution. Following this stringent wash, the strips were incubated with 2 ml of alkaline phosphatase-streptavidin conjugate for 30 min at room temperature. Strips were washed twice with 2 ml of rinse solution and once with 2 ml of substrate buffer. Two milliliters of substrate (5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium) were added and incubated for 30 min at room temperature. The reaction was stopped by washing for 3 and 10 min with rinse solution and by a wash with 2 ml of water. The strips were dried, and the purple colored bands were visually interpreted.

Gel electrophoresis. Gel electrophoresis was performed in 2.0% agarose gels in 0.5× Tris-acetate-EDTA buffer. The marker used was a 100-bp marker.

Sequence analysis. For sequence analysis of PM amplimers, fragments were excised from 3% Tris-borate-EDTA agarose gels and purified with the QIAquick gel extraction kit. Purified amplimers were directly sequenced according to the manual of the Big Dye terminator cycle sequencing kit using forward PM-PCR primers. The sequence products were subsequently read using the 3100-Avant Genetic Analyzer. The resulting DNA sequences were analyzed with the Vector NTI Advance 9.0 software and compared with all known HPV types present in the National Center for Biotechnology Information database utilizing nucleotide-nucleotide BLAST (blastn) (2) (http://www.ncbi.nlm.nih.gov/BLAST/).

HPV sequences from GenBank. The following accession numbers of HPV sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/index.html) and used as a reference for the corresponding HPV genotype: HPV type 5, M17463; HPV type 8, M12737; HPV type 9, X74464; HPV type 12, X74466; HPV type 14, X74467; HPV type 15, X74468; HPV type 17, X74469; HPV type 19, X74470; HPV type 20, U31778; HPV type 21, U31779; HPV type 22, U31780; HPV type 23, U31781; HPV type 24, U31782; HPV type 25, X74471; HPV type 36, U31785; HPV type 37, U31786; HPV type 38, U31787; HPV type 47, M32305; HPV type 49, X74480; HPV type 75, Y15173; HPV type 76, Y15174; HPV type 80, Y15176; HPV candidate type 92, AF531420; HPV93, AY382778; HPV candidate type 96, AY382779.

Intra- and interlaboratory reproducibility. The reproducibility of the PM-PCR RHA method was tested on clinical materials and plasmid dilution series in three steps.

The first step was to test the interlaboratory variability of the reverse hybridization part of the assay. This RHA was carried out on amplimers derived from 20 eyebrow hair samples from healthy individuals. After the DNA extraction with the QIAamp DNA mini kit, the PM-PCR was performed in one laboratory. The amplimers were sent to two other laboratories. In one laboratory (location I), the AUTO-LiPA was used, whereas in the other laboratory (location II), the assay was performed manually. As a means to calculate the reproducibility, the genotyping results of the samples were judged either as concordant (both results yield completely identical genotypes), compatible (both results show one or more of the same genotype[s]), or discordant (no HPV type[s] is [are] the same in both laboratories) (27).

In the second step, the analytical sensitivity of the PM-PCR RHA method was compared in two laboratories by testing dilution series for six beta-PV plasmid clones (representing HPV types 5, 8, 17, 22, 23, and 38). The 10-fold serial dilutions contained 1,000 to 0.1 copies of plasmid DNA and 50 ng of human genomic DNA as input for the PCR. The analytical sensitivity was measured as the copy number of the respective plasmid clones detectable in the reverse hybridization assay.

In the third step, the intra- and interlaboratory variation for the complete PM-PCR RHA method was analyzed. For this purpose, isolated DNA from random samples of an ongoing case control study of renal transplant recipients was used. DNA isolations of 45 eyebrow hair samples and 5 negative isolation controls were performed with the QIAamp DNA mini kit. These DNA samples were exchanged and tested twice in laboratories I and II. Reproducibility was measured in the same manner as described for step one.

RESULTS

PM-PCR. (i) Type specificity. DNA from 25 plasmids containing partial or complete HPV genomic sequences representing the whole established beta-PV genus were analyzed by PM-PCR. Amplimers of the expected size of 117 bp were obtained from all plasmids as determined by detection with gel electrophoresis. Subsequent sequence analysis (data not shown) confirmed that the correct genotypes were amplified.

(ii) Genus specificity. To determine the genus specificity of PM-PCR, the E1 sequences from 59 alpha-PVs, 6 gamma-PVs, 2 mu-PVs, and 1 nu-PV were aligned with the E1 sequences from the beta-PVs. HPV67 from the alpha genus and HPV65 from the gamma genus had the least number of mismatches with the PM primer set (7 and 6 mismatches with the best matching primer pair, respectively). Two million copies from each of these types were tested by PM-PCR. No amplimer was detected by both gel electrophoresis and reverse hybridization

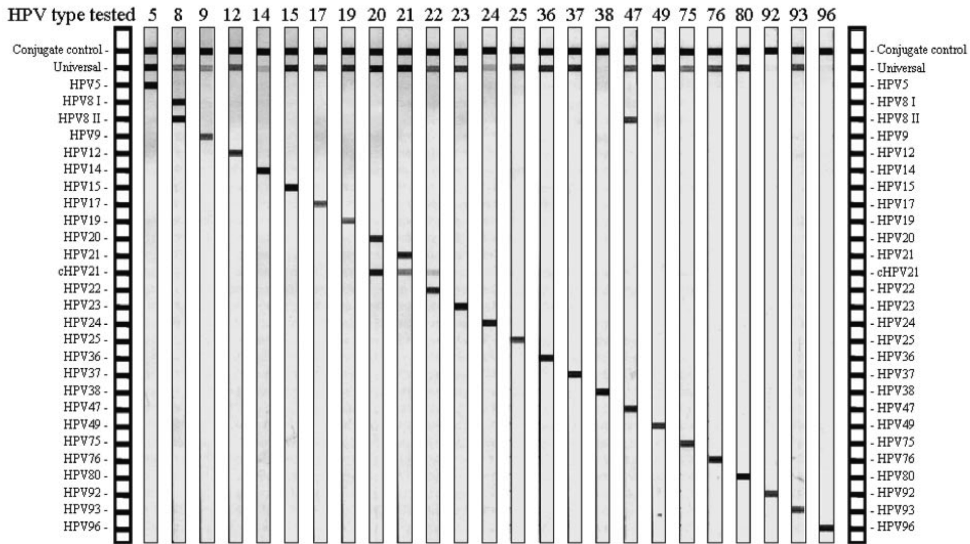

FIG. 2. Outline of the PM-PCR RHA method and typical patterns arising upon analysis of PM-PCR-derived amplimers. The top line is the conjugate control, which serves as the positive control for the enzymatic coloring reaction. The other lines are indicated by the probe names, beginning with the mixture of general probes ("Universal"). The remaining lines represent genotype-specific probes, except for probes HPV8 I, HPV8 II, HPV21, and cHPV21, which are used in pattern recognition of genotypes. The tested amplimers were obtained by performing the PM-PCR on plasmid clones of 23 beta-PV genotypes and 2 beta candPV genotypes (genotyping results representing HPV types 5, 8, 9, 12, 14, 15, 17, 19 to 25, 36 to 38, 47, 49, 75, 76, 80, cand92, 93, and cand96 are shown from, respectively, strips 1 to 25).

(data not shown). Genotypes from the other two HPV genera mu and nu were not tested, since these show at least 10 mismatches with any of the PM primers (data not shown).

(iii) Analytical sensitivity. Sensitivity tests were performed on 10-fold serial dilutions of plasmid clones of HPV types 5, 8, 9, 15, 17, 19, 23, 24, 36, 38, 47, 49, 93, and cand96. Plasmid dilution series were made in a background of human DNA as described in Materials and Methods. A typical example of the agarose gel electrophoresis of the products of such a dilution series is depicted in Fig. 3A.

The analytical sensitivity of the PM-PCR RHA ranges from 10 to 100 copies for beta-PV types 5, 8, 9, 15, 17, 19, 23, 24, 36, 38, 47, 49, 93, and cand96. Probe HPV8 I and HPV8 II detect HPV8 with a sensitivity of 100 and 10 copies, respectively.

Reverse hybridization assay: analytical specificity. To assess the efficacy and reliability of the RHA, 25 beta-PV sequences, representing the whole established genus, were amplified by PM primers from plasmids containing complete or partial beta-PV genomic sequences. Genotyping by direct sequencing (data not shown) of the obtained amplimers from the E1 region and analysis by RHA (Fig. 2) yielded the same expected HPV genotyping result in all 25 cases, indicating the high analytical specificity of the RHA.

Comparison of PM-PCR RHA with MaHa PCR assay. The analytical sensitivity of the PM-PCR RHA method was compared to an established beta-PV detection system, the MaHa PCR assay, a nested, broad-spectrum PCR method utilizing a mixture of degenerated primers. DNA was amplified from a

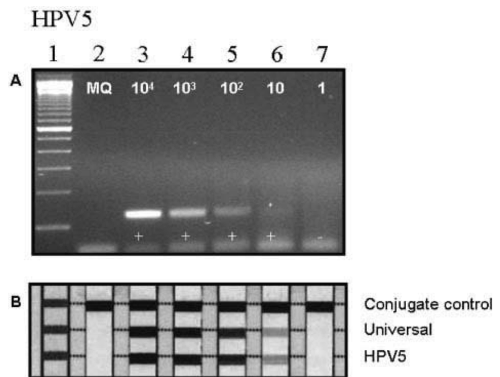

FIG. 3. (A) Typical example of the sensitivity range of the PM-PCR RHA method as demonstrated by input of an HPV5 serial plasmid dilution in a background of human genomic DNA (50 ng). Ten microliters of PM-PCR product was analyzed by electrophoresis. The PM-PCR mix control contained water (lane 2). DNA input in the PCR ranged from 10,000 (lane 3) to 1 (lane 7) copies of HPV5 plasmid DNA, with each lane starting from lane 3 representing a 10-fold dilution. Lane 1 contains a 100-bp DNA marker. (B) RHA results of the samples from panel A. The top line is a positive control containing biotinylated DNA, the second probe line is for general beta-PV detection, and the third probe line has the HPV5 type-specific probe.

TABLE 1. HPV detection results of a panel of 50 eyebrow hair samples tested with both the PM-PCR RHA method and the MaHa PCR

PM-PCR RHA method result	No. of samples with MaHa PCR result		Total no. of samples
	Positive	Negative	
Positive	40	8	48
Negative	1	1	2
Total	41	9	50

panel of 50 eyebrow hair samples selected from the Leiden Skin Cancer Study with the MaHa PCR assay and analyzed by gel electrophoresis. PM-PCR RHA results obtained from the same panel of isolated DNA samples were scored as either positive or negative for beta-PV. The PM-PCR RHA method revealed a positive result for 48 of the 50 (96%) samples (Table 1). With the MaHa PCR assay, 41 of the 50 (82%) samples were positive. In one of the PM-PCR RHA-negative samples, the MaHa PCR was positive. The detection rate of the PM-PCR RHA method is significantly higher than the detection rate with the MaHa PCR ($P = 0.019$, chi-square test). In addition, the PM-PCR RHA system could identify the beta-PV genotypes present in the sample. The number of types present ranged from 1 type in 32% of the cases up to 2 to 10 types in 64% of the samples (the average number of types in the positive samples was 3.5). The PM-PCR RHA system evidently has a higher analytical sensitivity for the detection of beta-PV genotypes in plucked hairs and, furthermore, allows the simultaneous identification of multiple genotypes. The difference in analytical sensitivity is not due to a higher sensitivity of the RHA system over agarose gel electrophoresis (as is shown in Fig. 3A and B).

Performance of the PM-PCR RHA method on paraffin-embedded materials. The positivity rate for beta-PV types was 80% in formalin-fixed paraffin-embedded shaved skin biopsy specimens ($n = 20$) (Table 2).

Intra- and interlaboratory reproducibility. (i) RHA. The reproducibility of the PM-PCR RHA method was tested on clinical materials and plasmid dilution series in three steps. The first step was to test the interlaboratory variability of the reverse hybridization part of the method on amplimers derived from 20 eyebrow hair samples from healthy individuals. Amplimers were generated by PM-PCR by one laboratory and were genotyped by reverse hybridization in two other laboratories (Table 3). For 11 samples (55%), the RHA showed concordant (identical) HPV genotyping results, and in 8 samples (40%), a compatible result was obtained, meaning that at least one of the detected genotypes was found by both laboratories. A discordant result was observed in one sample. The overall reproducibility was 95% when concordant and compatible results were combined.

(ii) PM-PCR RHA analytical sensitivity. In the next step, the reproducibility of the analytical sensitivity of the PM-PCR RHA method was determined by testing six serial 10-fold plasmid dilution series of HPV types 5, 8, 17, 22, 23, and 38 in two different laboratories. Overall, the analytical sensitivity found by both laboratories was 10 to 100 copies. At both laboratories, identical detection limits were found for HPV types 5, 8, 17, 23, and 38. Only in the case of HPV type 22 did the analytical sensitivity varied from 10 copies in one laboratory to 100 copies in the other.

TABLE 2. Genotyping results obtained from 20 paraffin-embedded shaved normal skin biopsy specimens from cutaneous SCC patients tested by the PM-PCR RHA method

Sample	HPV type(s)[a]
1	—
2	76, 93
3	23, 24, 36, 37, 49
4	24
5	—
6	—
7	—
8	15, 20
9	80
10	15, 37
11	20, 23, 24, 76
12	19, 20, 23, 24
13	5, 22, 23
14	19, 23
15	12, 15, 17, 19, 22
16	19, 23, 36, 37, 38
17	5
18	5, 20, 24, 75, 92
19	23, 24, 25, 76, 80, 92
20	23, 36, 96

[a] —, no beta-PV type detected.

TABLE 3. Interlaboratory reproducibility of the RHA in a panel of 20 PCR products. The PCR products were obtained from eyebrow hair samples from healthy individuals

Sample	HPV type(s) determined by RHA at location:		Reproducibility[a]
	I	II	
1	23	23	i
2	23, 93	23, 38, 93	c
3	8, 20, 23, 38, 49, 92	8, 20, 23, 38, 49, 92	i
4	—[b]	36	d
5	23, 80	23, 80	i
6	12, 14, 19, 23	12, 14, 19, 23	i
7	12, 14, 19, 23, 25	12, 14, 15, 19, 23, 25	c
8	14, 17, 38, 93	14, 38, 93	c
9	12, 23, 24, 80	12, 23, 24, 80	i
10	14, 19	14, 19	i
11	—	—	i
12	5, 17, 23, 93	5, 23, 93	c
13	8, 9, 15, 20, 23, 38, 49, 92	8, 15, 20, 23, 38, 49, 92	c
14	19, 38	19, 38	i
15	80	80	i
16	8, 38	8, 38	i
17	23	23, 38	c
18	—	—	i
19	12, 93	5, 8, 12, 93	c
20	12, 17, 23, 24, 80	5, 12, 15, 17, 23, 24, 25, 36, 37, 47, 80, 93	c

[a] The interlaboratory reproducibility is divided into concordant results (i, both results are identical), compatible results (c, both results show at least one or more of the same genotype[s]), and discordant results (d, no similarities are found between both results).
[b] —, no beta-PV type detected.

TABLE 4. Panel of 50 eyebrow hair samples tested on two different locations by the PM-PCR RHA method twice

Sample	Location I			Location II		
	HPV type(s) determined by PM-PCR RHA		Reproducibility[a]	HPV type(s) determined by PM-PCR RHA		Reproducibility
	Test 1	Test 2		Test 1	Test 2	
1	9, 36	36	c	36	36	i
2	24, 92, 93, 96	24, 93, 96	c	23, 24, 92, 93, 96	23, 24, 92, 93, 96	i
3	8	8, 23	c	8	8	i
4	8, 23, 49, 76, 93	8, 23, 24, 49, 76, 93	c	8, 23, 49, 76, 93	8, 23, 24, 49, 76, 93	c
5	9, 24	9, 24	i	8, 9, 24	9, 24	c
6	15, 38	15, 24, 38	c	15, 38	15, 38	i
7	20, 38, 75	20, 38, 49, 75	c	20, 38, 75, 93	20, 38, 75, 93	i
8	12, 21, 75	12, 38, 75	c	12, 23, 75	12, 23, 38, 75	c
9	5, 9, 23, 36, 37, 38, 92	5, 9, 23, 36, 37, 38, 92	i	5, 8, 23, 36, 37, 38, 92	8, 23, 36, 37, 38, 92	c
Control[b]	—[c]	—		—	—	
11	5, 9, 23, 36, 92	9, 23, 36, 92	c	9, 23, 36, 92	9, 23, 36, 92	i
12	5, 15, 23, 36, 80	5, 23, 36, 80	c	5, 23, 36, 80	5, 15, 23, 36, 80, 93	c
13	5, 8, 20, 21, 24, 37, 38, 76	5, 8, 20, 21, 24, 37, 38, 76	i	5, 8, 20, 24, 37, 38	5, 8, 20, 24, 37, 38	i
14	5, 8, 9, 14, 19, 20, 23, 24, 75	5, 8, 9, 14, 19, 20, 23, 24, 75	i	5, 14, 15, 19, 20, 23, 24, 75	5, 14, 19, 20, 23, 75	c
15	25	8, 25	c	8, 25	8, 20, 24, 25	c
16	—	23	d	23	—	d
17	8, 9, 15, 22, 23, 24, 92, 93	8, 9, 15, 17, 22, 23, 24, 92, 93	c	8, 9, 15, 22, 23, 24, 92, 93	8, 9, 15, 22, 23, 24, 92, 93	i
18	5, 96	5, 96	i	5, 96	5, 80, 93, 96	c
19	24, 93	24, 75, 93	c	24, 93	24, 93	i
Control	—	—		—	—	
21	5, 24, 38	5, 24, 38	i	5, 24, 38	5, 24, 38	i
22	24, 38	24, 38, 75	c	24, 37, 38	24, 37, 38	i
23	8, 9, 19, 23, 24, 80	8, 9, 23, 24, 80	c	8, 23, 80	8, 23, 80	i
24	38, 49	49	c	49	38, 49	c
25	5, 9, 14, 23, 93	5, 8, 9, 14, 15, 22, 23, 93	c	14, 23, 93	5, 9, 14, 23	c
26	23, 24	23	c	23	23	i
27	23, 24, 37, 49	23, 37, 38, 49	c	23, 24, 37, 49	23, 37, 49	c
Control	—	—		—	—	
29	9, 15, 23	9, 15, 17, 23, 49	c	9, 15, 23	9, 15, 23	i
30	9, 17, 24, 36	5, 8, 9, 17, 19, 23, 24, 36, 38, 75, 76	c	5, 8, 9, 17, 23, 24, 36, 38, 75	5, 8, 9, 23, 24, 36, 38, 75	c
31	—	—	i	—	—	i
32	—	9, 12, 15, 23	d	9, 12, 15, 23	9, 12, 15, 23	i
33	5, 20, 23, 24, 36, 38, 92, 96	20, 23, 24, 36, 38, 49, 92, 96	c	5, 20, 23, 24, 36, 38, 96	5, 8, 12, 20, 23, 24, 36, 38, 92, 96	c
34	5, 38, 49, 76, 92	5, 8, 38, 49, 76, 92	c	5, 38, 49, 76, 92	5, 38, 49, 76, 92	i
35	5, 23, 24, 25	5, 19, 24, 25, 49	c	5, 24	8, 9, 14, 15, 19, 23, 24, 25	c
36	5, 8, 9, 23, 24, 96	5, 8, 9, 17, 23, 24, 96	c	5, 9, 17, 23, 24, 96	5, 8, 9, 17, 23, 24, 96	c
37	22, 24, 36, 49, 93, 96	22, 24, 36, 38, 49, 93, 96	c	22, 24, 36, 49, 93, 96	22, 24, 36, 49, 93, 96	i
Control	—	—		—	—	
39	5, 8, 15, 24, 38	5, 8, 15, 24, 38	i	15, 24, 38	8, 15, 23, 24, 38	c
40	8, 15, 22, 23	8, 22, 23	c	8, 23	8, 22, 23	c
41	5, 15, 23, 24, 36, 37, 49, 76	5, 15, 23, 37	c	23	5, 15, 23, 24, 36, 37, 49, 76	c
42	5, 8, 15, 19, 20, 23, 36, 37, 38, 49	5, 8, 15, 19, 20, 23, 36, 37, 38, 49	i	5, 15, 19, 20, 23, 36, 37, 38, 49	8, 15, 19, 20, 23, 24, 36, 37, 38, 49	c
43	5, 9, 23, 24	5, 9, 23, 24	i	5, 9, 23, 24	5, 9, 23, 24	i
44	8, 23	8, 19, 23	c	19, 23, 24	8, 19, 23	c
45	5, 9, 15, 23, 24, 37, 38, 93	5, 9, 23, 24, 37, 38, 92, 93	c	5, 9, 23, 24, 37, 38, 93	5, 9, 15, 23, 24, 37, 38, 93	c
46	—	5, 8, 12, 20, 23, 36	d	5, 8, 12, 20, 23, 36	5, 8, 12, 20, 23, 36	i
47	38	—	d	38	38	i
Control	—	—		—	—	
49	8, 23, 24, 36, 96	8, 23, 24, 36, 96	i	8, 23, 24	8, 14, 23, 24, 36, 38, 96	c
50	5, 12, 14, 24, 92, 93	5, 8, 12, 14, 24, 92, 93, 96	c	5, 12, 14, 24, 93	8, 12, 14, 24, 93	c

[a] The interlaboratory reproducibility is divided into concordant results (i, both results are identical), compatible results (c, both results show at least one or more of the same genotype[s]), and discordant results (d, no similarities are found between both results).
[b] The negative isolation controls are designated control and contain 100 ng of human genomic DNA.
[c] —, no beta-PV type detected.

TABLE 5. Comparison of genotyping results from the panel of 45 eyebrow hair samples from Table 4

Reproducibility comparison	% Concordant	% Compatible	% Discordant	% Reproducibility[a]	Agreement beyond chance for[b,c]:				
					HPV5	HPV8	HPV23	HPV24	HPV38
Intralaboratory									
Location I.1 vs location I.2	24	67	9	91	0.82 (0.64–0.99)	0.71 (0.49–0.93)	0.74 (0.54–0.93)	0.87 (0.72–1.00)	0.69 (0.46–0.92)
Location II.1 vs location II.2	47	51	2	98	0.79 (0.60–0.99)	0.61 (0.36–0.85)	0.86 (0.71–1.00)	0.69 (0.47–0.90)	0.85 (0.68–1.00)
Interlaboratory									
Location I.1 vs location II.1	31	62	7	93	0.72 (0.51–0.93)	0.43 (0.12–0.74)	0.69 (0.48–0.90)	0.82 (0.66–0.99)	0.89 (0.75–1.00)
Location I.1 vs location II.2	36	60	4	96	0.62 (0.37–0.86)	0.61 (0.36–0.85)	0.74 (0.54–0.93)	0.69 (0.48–0.90)	0.85 (0.68–1.00)
Location I.2 vs location II.1	29	69	2	98	0.81 (0.64–0.99)	0.51 (0.24–0.78)	0.82 (0.65–0.99)	0.78 (0.59–0.96)	0.79 (0.60–0.99)
Location I.2 vs location II.2	29	67	4	96	0.71 (0.50–0.93)	0.72 (0.52–0.93)	0.73 (0.52–0.93)	0.73 (0.53–0.93)	0.75 (0.55–0.96)
Overall (location I 1 & 2 vs location II 1 & 2)	13	87	0	100					

[a] To measure reproducibility, the percentages of concordant and compatible results were added together. Negative isolation controls were not included in the calculation of these results.
[b] To measure reproducibility, the agreement beyond chance (kappa) for the 5 most frequently encountered HPV types was calculated. The 95% confidence intervals are indicated in parentheses. Negative isolation controls were not included in the calculation of these results.
[c] The average detection rates in the panel (45 samples) were 37%, 33%, 55%, 47%, and 32% for HPV5, 8, 23, 24, and 38, respectively.

(iii) PM-PCR RHA method. In the third step, a panel containing isolated DNA from random samples of an ongoing case control study of renal transplant recipients was selected. DNA of 45 eyebrow hair samples and 5 negative isolation controls was isolated with the QIAamp DNA mini kit. The isolated DNA was tested twice by the PM-PCR RHA method on the two different locations to determine intra- and interlaboratory reproducibility. The genotyping results are shown in Table 4. All negative isolation controls remained negative in the quadruple tests. Of the samples, 98% were found to harbor beta-PV DNA. The number of beta-PV types identified per sample varied from 1 type to 11 types (the average of types per sample was 4.4). All known beta-PV types were found except HPV type 47. Only one sample (sample 31) remained negative in all four tests. Two samples were once negative and the other three times found to contain DNA from four or more concordant beta-PV types (Table 4, samples 32 and 46). Two other samples showed the presence of one or no beta-PV type (Table 4, samples 16 and 47).

The intra- and interlaboratory reproducibility was calculated and is shown in Table 5. Intralaboratory analysis revealed that the percentage of concordant results varied from 24% to 47%, whereas the percentage of compatible results varied from 51% to 67%. Discordant results were observed in 2% to 9% of the samples. Thus, the intralaboratory reproducibility varied from 91% to 98%.

The interlaboratory reproducibility varied from 93% to 98%, i.e., the percentage of concordant results varied from 29% to 36%, whereas the percentage of compatible results varied from 62% to 69%. The percentages of discordant results ranged from 2% to 7%.

When the results obtained per sample in each laboratory are compared to the results obtained for that sample in the other laboratory, the percentage of concordant results is 13% and the percentage of compatible results is 87%. Thus, taken together, the overall reproducibility is 100%. The intralaboratory and interlaboratory agreement beyond chance (kappa) varied for the 5 most frequently encountered genotypes in the panel (i.e., HPV5, HPV8, HPV23, HPV24, and HPV38) from 0.43 to 0.89 (Table 5).

DISCUSSION

Detection of HPV DNA. Multiple broad-spectrum PCRs that predominantly target the L1 gene have already been developed for the detection of the beta-PV genus. In this study, we describe a novel highly sensitive single-step broad-spectrum PCR targeting the E1 region combined with a reverse hybridization assay for the detection and identification of the beta-PV genus genotypes. The analytical sensitivity of the assay varied between 10 and 100 copies of HPV DNA in a human genomic DNA background. We compared the analytical sensitivity of the novel approach with the MaHa PCR, a nested beta-PV-specific PCR using a mix of degenerated primers (3, 5, 11). The nested MaHa PCR has several disadvantages. First, use of a nested PCR gives a higher risk of contamination than that of a one-step PCR. Secondly, degenerated primer batches show batch-to-batch variation of primer composition, resulting in a decrease in reproducibility (14). Another shortcoming is the relatively low analytical sensitivity (Table 1). This is probably due to the large target of 779 bp amplified in the first step of the MaHa PCR. Consequently, the MaHa PCR is likely to be less efficient in the amplification of DNA from paraffin-embedded formalin-fixed patient material than the PM-PCR because of the large size of the (first step) PCR product, which is disadvantageous in such materials (17).

The above mentioned disadvantages are circumvented with the use of the PM-PCR, since it is a one-step PCR that uses nondegenerated primers and amplifies only a small fragment of 117 bp. Due to the small amplimer size, the assay is able to

amplify low-quality DNA from formalin-fixed, paraffin-embedded materials, which is an important advantage.

Furthermore, the PM-PCR was found to be specific for only the beta-PV genus, probably because the number of mismatches between the primer set and nucleotide sequences from other HPV genera is at least six.

The problem common to all broad-spectrum PCR primer-mediated PCR methods is the competition between the different HPV types present in one sample. For example, if an HPV type is present in great molar excess over another type, it is possible that this last type will be out competed and would escape detection in this kind of assay (27). This underestimation of types has also been described for other broad-spectrum PCR systems like SPF10 and PGMY (27).

HPV genotyping. Several methods are available for identification of beta-PV genotypes in clinical samples including, (i) the use of type-specific PCRs (23); (ii) direct sequencing or cloning and sequencing of amplimers derived from broad-spectrum PCRs (5); (iii) restriction fragment length polymorphism analysis of amplimers (18); and (iv) dot blot assay using type-specific labeled oligonucleotide probes (1). With these testing methods, reliable results can be achieved. However, when in large epidemiological studies simultaneous detection and identification of all the 25 beta-PV types is required, these methods are very laborious and can lead to an underestimation of the number of types present. With type-specific PCR, a separate amplification reaction is required for every type, which is not only time-consuming but also requires large amounts of input DNA.

Typing by cloning and sequencing of broad-spectrum PCR-derived amplimers is a very accurate method. However, as is shown in Table 4, mixed infections of beta-PV types are regularly found in single samples. The use of the cloning and sequencing strategy would mean that a very high number of clones must be tested to ensure the identification of all present beta-PV types. However, an advantage of the sequencing approach is that new HPV types can be found more often than with the PM-PCR RHA method.

With the newly developed beta-PV genotyping assay, it is possible to test for 25 HPV types in one reaction. This is a significant improvement of the presently available techniques, especially when a large number of samples has to be tested.

Performance of the assay. When large epidemiological studies have to be performed to determine the association between the presence of specific HPV types and (pre)malignant skin lesions, it is desirable to use a very sensitive and fast assay to detect the DNA of the various types. The PM-PCR RHA method meets these requirements, since it is fast and sensitive, as it is able to detect 10 to 100 copies of beta-PV plasmid DNA per PCR. The analytical sensitivity for the detection does not seem to vary more than 10-fold between the different beta-PV types. This might help in establishing possible associations between certain beta-PV types and clinical disease more accurately.

In the clinical samples tested in this study, all established beta-PV types were found except for HPV47. The testing of an HPV47 plasmid dilution series showed that this genotype could be detected with a sensitivity of 10 copies, showing that the lack of detection of HPV47 is most likely not caused by a low sensitivity of the PM-PCR RHA method. As stated in Materials and Methods, HPV8 is recognized by probes HPV8 I and HPV8 II. Probe HPV8 II has a higher sensitivity than probe HPV8 I, but it cannot be used for HPV8 identification if HPV47 is present. This leads to a limited ability to detect HPV8 if it is present at copy numbers lower than 100 copies in combination with HPV47.

The reproducibility was carefully examined at three levels. The first level was to exchange PCR products and to study the variation of the reverse hybridization assay in an interlaboratory setting. The results showed high reproducibility (Table 3). In the next level, the performance of the PM-PCR RHA method was tested on two different locations by analyzing dilution series for six beta-PV plasmid clones. No significant differences in analytical sensitivity were observed, indicating that the PCR performed equally well in both laboratories. The third level showed highly reproducible results for the analysis of 50 samples of isolated plucked eyebrow hair DNA and negative isolation controls in intra- and interlaboratory settings (Table 4 and 5). The intra- and interlaboratory agreement beyond chance (kappa) for the 5 most frequently encountered HPV types was calculated. For HPV5, 23, 24, and 38, this agreement was good to very good. The interlaboratory agreement for HPV8 was moderate to good, and the intralaboratory agreement was good. This indicates that the assay is robust.

As expected, the percentage of concordant results is the highest for the panel of exchanged PCR products. Overall, the data indicate the high reproducibility of both the PM-PCR and the RHA.

The reproducibility is not 100%, as can be expected when using PCR-based methods. For example, in samples with a very low viral load, sampling variation may play an important role, since not every aliquot taken from this sample will contain sufficient HPV molecules of a certain type to permit detection by the PCR. Thus, samples containing only a low viral load will sometimes yield false-negative results, given that detection in such aliquots is based on pure chance.

Since a high number of samples contain multiple HPV types, the competition between primers and targets may cause an underestimation of the number of types present in a sample. When one genotype is present in great molar excess over another genotype, the broad-spectrum PCR and RHA will only detect the major genotype. However, it has been shown previously that RHA is much more sensitive in detecting minority genotypes than. e.g., direct sequence analysis (20). For sequence analysis, a genotype has to represent at least 25% of the total DNA, whereas the RHA can sometimes detect minority genotypes at a level of less than 1% input in the PCR mixture (unpublished data).

The chance of contamination is an important issue due to the high analytical sensitivity of the system and the ubiquity of the beta-PV types on normal human skin. Desquamated skin cells infected with beta-PV types can easily be distributed in the laboratory. Therefore, special measures have to be taken to perform beta-PV PCR testing. Although there is no evidence that contamination played a role in the described experiments (e.g., the negative isolation controls in Table 4), it must be monitored closely.

Finally, the present method aims at the identification of the 25 known beta-PV genotypes. However other beta-PV types exist which have not been fully characterized so far. These might also react with the primers and probes of the current

assay and may yield aberrant results. At the same time, the assay contains general detection probes, which may serve to identify such novel genotypes.

To show the accuracy of the typing results obtained with the PM-PCR RHA method, further studies will have to be done. For instance, it would be desirable to compare the PM-PCR RHA method, targeting the E1 gene, with another genotyping assay also capable of multiparameter testing but then targeting another part of the viral genome, like the RLB assay, which was recently published (8).

In conclusion, the PM-PCR RHA method for the detection of 25 beta-PV types is highly sensitive and reproducible. Therefore, it is a very useful tool for the identification of beta-PV, especially in large epidemiological studies aimed at investigating the association between individual HPV types and cutaneous (pre)malignant lesions.

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