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## Evaluation of a Novel Broad-Spectrum PCR–Multiplex Genotyping Assay for Identification of Cutaneous Wart-Associated Human Papillomavirus Types<sup>∇</sup>

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**A large number of human papillomavirus (HPV) types, distributed over five papillomavirus genera, are detectable in the skin. HPV types belonging to the alpha, gamma, and mu genera have been detected in cutaneous warts. A state-of-the-art HPV genotyping assay for these cutaneous wart-associated HPV types does not exist although warts constitute a highly prevalent skin condition, especially in children (33%) and organ transplant recipients (45%). Cutaneous warts are again the focus of attention as their clinical relevance rises with the increasing number of chronically immunosuppressed patients. The objective of this study was to develop and evaluate a DNA-based genotyping system for all known cutaneous wart-related HPV types using PCR and Luminex xMAP technology. The broad-spectrum PCR amplified DNA of all known wart-associated HPV types from the genera alpha (HPVs 2, 3, 7, 10, 27, 28, 29, 40, 43, 57, 77, 91, and 94), gamma (HPVs 4, 65, 95, 48, 50, 60, and 88), mu (HPVs 1 and 63), and nu (HPV41). The probes were evaluated using plasmid HPV DNA and a panel of 45 previously characterized cutaneous wart biopsy specimens showing high specificity. HPV was also identified in 96% of 100 swabs from nongenital cutaneous warts. HPV types 1, 2, 27, and 57 were the most prevalent HPV types detected in 89% of the swabs. In conclusion, this Luminex-based genotyping system identifies all known cutaneous wart HPV types including phylogenetically related types, is highly HPV type specific, and is suitable for large-scale epidemiological studies.**

Papillomaviruses (PV) constitute a group of viruses associated with benign and malignant proliferative lesions of cutaneous and mucosal epithelia. The taxonomic family of PV includes 16 genera (5). More than 100 human papillomavirus (HPV) types have been fully sequenced. The number of HPV types that are not yet fully characterized is probably much larger. HPV types, distributed over five genera (alpha, beta, gamma, mu, and nu) and 16 species, infect the skin (5). HPV types belonging to species of three genera (alpha, gamma, and mu) have most frequently been detected in hyperkeratotic skin lesions (warts).

The alphapapillomavirus (alpha-PV) types infecting the genital mucosa (e.g., HPV16, HPV18, HPV6, and HPV11) are the best understood. HPV16 and HPV18 are the most prevalent types involved in the pathogenesis of anogenital (e.g., cervical) cancer, and HPV types 6 and 11 cause genital warts and laryngeal papillomas. The alpha-PV types belonging to species 2, 4,

and 8, which have been detected in cutaneous warts, have been studied less thoroughly. At present the most frequently detected alpha-PV types in cutaneous warts are HPV types 2, 3, 10, 27, and 57 (3, 10, 12–16, 18, 19). Gamma-PV types, including HPV4, HPV60, and HPV65, have also regularly been detected in cutaneous warts (4, 10, 12, 15). Mu-PV types, HPV1 and HPV63, have also been detected in cutaneous warts (7). HPV types included in the beta and nu genera have rarely been detected in cutaneous warts. Only Harwood and coworkers (13) have described the presence of beta-PV types in cutaneous warts of immunosuppressed patients. The nu-PV genus consists of only one HPV type (HPV41), which was originally isolated from a facial wart (11).

In general, cutaneous warts from immunocompetent and immunocompromised patients appear to show the same genotype distributions (16, 19). This has to be confirmed by a large-scale study comparing the distributions in the two populations with the same techniques. The number of detected types per lesion appeared to differ between immunocompetent and immunosuppressed patients (13). Currently, cutaneous warts are again the focus of attention with the increasing number of chronically immunosuppressed patients (9, 20). Signifi-

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cant physical and psychological morbidity in these patients may result from the existence of confluent plaques of warts present on cosmetically sensitive areas such as the face and hands and the presence of large warts occurring on pressure-bearing areas such as the feet.

Although cutaneous warts constitute a highly prevalent skin condition, especially in children (33%) (22) and organ transplant recipients (45%) (2), only a few large-scale epidemiological studies including more than 100 lesions have investigated the distribution of HPV types in cutaneous warts. These studies utilized different detection methods, such as general primer-mediated PCR followed by HPV typing with direct sequencing (12, 15) or by restriction enzyme cleavage of the PCR product (18). Other methods employed were multiple-type-specific PCR combined with direct sequencing (3), *in situ* hybridization (8), and (Southern) blot hybridization (6, 10). Although these methods are in general type specific, they are time-consuming and therefore less suitable for large scale-epidemiological studies. In the case of multiple infections, reliable typing might also be difficult with direct sequencing of the PCR amplicon without prior molecular cloning. Therefore, we have evaluated a novel broad-spectrum PCR–multiplex genotyping (MPG) assay called the hyperkeratotic skin lesion (HSL) PCR/MPG for identification of established and candidate cutaneous wart HPV types. These types are included in the alpha genus species 2 (HPVs 3, 10, 28, 29, 77, and 94), species 4 (HPVs 2, 27, and 57), and species 8 (HPVs 7, 40, 43, and 91); gamma genus species 1 (HPVs 4, 65, and 95), species 2 (HPV48), species 3 (HPV50), species 4 (HPV60), and species 5 (HPV88); mu genus species 1 (HPV1) and species 2 (HPV63); and nu genus species 1 (HPV41). In this study the sensitivity, the HPV type specificity, and the clinical performance of the HSL-PCR/MPG broad-spectrum assay have been investigated.

**MATERIALS AND METHODS**

**Clinical materials.** Two panels of clinical samples were available. The first panel consisted of purified DNA obtained from 45 nongenital cutaneous warts that were taken from immunocompetent patients, immunosuppressed organ transplant patients, and HIV-positive patients. These samples had been previously HPV genotyped by DNA sequencing using a purified PCR product from two rounds of nested PCR utilizing the primer pair HVP2-B5 for the first round and the pairs CN1F-CN1R, CN2F-CN2R, and CN3F-CN3R for the second round (sequencing) or the single-round primer pair C4F-C4R (PCR and sequencing), as described by Harwood and coworkers (13). The use of separate reaction mixtures with different primer pairs allowed the identification of multiple HPV types. In some cases DNA sequencing was not carried out with all of the primer pairs (Table 1).

A second panel comprised swabs taken from 100 cutaneous warts derived from 100 individuals (age of >4 years) with one or more cutaneous warts who self-presented to their general practitioners. The warts were present mainly on hands and feet. The swabs were taken from one wart from each patient by firmly rubbing a prewetted cotton-tipped stick five times over the surface of the lesion. Next, the swabs were put in 1 ml of saline solution, and 10 µl was used directly in the novel HSL-PCR/MPG assay.

**Plasmids.** For the evaluation of the analytical type specificity of the HSL-PCR/MPG assay (Labo Biomedical Products BV, Rijswijk, Netherlands), amplicons were generated using plasmid clones containing sequences of the following types: HPV types 1, 2, 3, 4, 7, 10, 27, 28, 29, 40, 41, 43, 48, 50, 57, 60, 63, 65, 77, 88, 91, 94, and 95. Plasmids containing HPV genomic DNA were kindly provided by E.-M. de Villiers (HPVs 1, 4, 7, 41, 48, and 63), O. Forslund (HPV88), M. Favre (HPV10), T. Matsukura (HPV60), R. Ostrow (HPV3), G. Orth (HPVs 28, 29, and 50), and A. Lorincz (HPV43). For HPV types 2, 27, 40, 57, 65, 77, 91, 94, and 95, the relevant HPV sequence was synthesized, cloned, and sequenced (GenScript USA, Inc.). In order to confirm amplification of an HPV-specific fragment, gel electrophoresis was performed with 2.2% FlashGel agarose gels

TABLE 1. Comparison of HSL-PCR/MPG assay results with direct DNA sequencing on PCR amplicons obtained from purified DNA from 45 warts

Sample no.	HPV type(s) by:		Comparison of results <sup>f</sup>
	HSL-PCR/MPG	DNA sequencing	
1	27	27	I
2	27	27	I
3	27	27, 94	C
4	27	27, 94	C
5	27	27	I
6	28	28	I
7	27	27	I
8	2	2	I
9	Negative	Negative	I
10	27	27	I
11	(3) <sup>a</sup> , 7	3	C
12	(3) <sup>a</sup> , 7	3	C
13	27	27	I
14	2	2	I
15	57	57	I
16	57	57	I
17	57	28, 57	C
18	57	57	I
19	57	57	I
20 <sup>b</sup>	57	72	D
21 <sup>b</sup>	Negative	72	I
22 <sup>c</sup>	4	27, 28	D
23	27	27, 28	C
24	57	57	I
25	27	27	I
26	27	27	I
27	27, 57, 95	27	C
28	27, 57	27	C
29	57	57	I
30	57	57	I
31	1, 3, 57	3	C
32	3, 57	57	C
33	57	57	I
34	4	4	I
35	2	2	I
36	2	2	I
37 <sup>d</sup>	7	10	D
38	4, 7, 10	7, 10, 27	C
39	4, 7, 10	7, 10, 27	C
40	7	7, 10	C
41	7, 10	10, 27	C
42	10, 27	27	C
43 <sup>e</sup>	63	27	D
44	27	27	I
45	4	4	I

<sup>a</sup> Elevated signal but below cutoff for HPV3 with the HSL-PCR/MPG. There was no sequencing result for HPV7; HPV7 was confirmed by a third assay.

<sup>b</sup> HPV72 not included in the HSL-PCR/MPG assay.

<sup>c</sup> No sequencing result for HPV4 available.

<sup>d</sup> Repeated DNA sequencing negative.

<sup>e</sup> Repeated DNA sequencing did not confirm HPV27.

<sup>f</sup> I, both methods yielded completely identical genotyping results (concordant); C, both methods showed one or more of the same genotype(s) (compatible); D, no similarity between genotypes detected by both methods (discordant).

(Lonza, Basel, Switzerland). To determine the analytical sensitivity of the assay, DNA concentrations of the plasmids were determined using a NanoDrop 2000 instrument (Thermo Fisher Scientific Inc.), and 10-fold serial dilutions were made in a background of 12 ng/µl human genomic DNA. This background human DNA is equivalent to approximately 24,000 cells per 10 µl of plasmid sample.



probe	HPV type																							
	alphaPV-2						alphaPV-4				alphaPV-8				gammaPV					muPV		nuPV		
	3	10	28	29	77	94	2v	2	27	57	7	40	43	91	4	48	50	60	65	88	95	1	63	41
conj	8601	9061	9095	8883	8690	8827	9016	9041	8653	8853	8559	8859	8436	8961	8822	8675	8891	8895	8276	9097	8774	8421	9172	9097
HPV3	5307	3	2	11	2	2	4	3	2	2	3	3	3	2	2	2	2	2	3	3	2	4	2	2
HPV10	17	5988	4	2	2	29	4	3	2	2	2	4	2	2	2	2	2	4	3	2	3	2	2	2
HPV28	15	16	2202	5	4	4	5	4	4	4	4	5	4	4	4	4	4	4	4	4	4	4	4	4
HPV29	5	6	6	3867	6	6	7	6	6	5	6	7	6	6	6	6	5	6	6	6	6	7	6	5
HPV77	3	3	3	43	5707	3	4	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
HPV94	3	13	11	3	3	2333	5	3	4	3	4	5	4	4	4	3	3	4	3	4	4	4	3	3
HPV2v	3	3	3	3	3	3	6742	101	43	6	3	4	3	3	3	3	3	3	3	3	3	3	3	3
HPV2	3	3	3	3	3	3	191	2780	4	3	3	3	4	3	3	3	3	3	4	3	3	3	3	3
HPV27	3	3	6	3	3	3	5	10	6666	3	3	4	4	4	3	3	4	3	3	3	3	3	3	3
HPV57	4	4	5	4	3	4	6	4	4	2768	3	5	4	4	4	4	4	5	5	5	4	5	4	4
HPV7	4	3	3	4	12	4	5	4	4	3	7667	5	4	4	4	3	4	4	4	3	4	4	3	4
HPV40	3	3	4	4	3	4	5	4	4	4	4	7340	3	4	3	3	3	3	4	4	4	4	3	3
HPV43	5	32	5	4	4	5	6	4	4	4	12	5	5562	10	4	4	4	4	5	4	5	4	4	4
HPV91	4	4	5	4	4	5	6	5	4	4	4	5	5	2477	5	5	5	5	5	4	5	5	4	5
HPV4	11	11	12	12	11	11	14	10	12	13	11	13	11	11	4379	11	12	13	23	12	13	13	12	11
HPV48	4	5	5	5	4	5	6	5	5	5	4	6	4	5	5	4178	5	5	5	4	5	5	5	4
HPV50	5	5	5	5	5	5	6	4	5	5	5	6	5	5	4	4	2133	6	6	5	5	5	9	5
HPV60	5	5	6	4	5	5	6	5	5	5	6	4	4	5	4	5	4196	5	5	5	5	5	4	5
HPV65	4	4	5	5	5	4	7	4	4	4	5	7	4	4	4	4	5	2334	5	8	5	4	5	5
HPV88	4	4	4	4	4	4	5	5	4	4	4	6	4	4	4	4	4	4	5	5489	4	4	4	4
HPV95	6	5	5	5	5	5	5	6	5	5	6	6	6	5	6	5	5	5	6	6	6888	6	5	5
HPV1	5	5	5	6	5	6	7	6	5	5	7	7	5	6	5	5	6	5	6	5	6	5396	6	5
HPV63	5	5	6	5	4	6	7	5	5	5	5	7	4	26	6	5	5	5	5	5	5	5	5983	4
HPV41	6	6	6	6	6	8	6	6	6	6	7	6	7	5	6	5	6	7	6	6	7	6	3601	

FIG. 2. Analytical type specificity of the HSL-PCR/MPG. Indicated are the MFI readouts (without background subtraction) of HSL-PCR amplimers generated from  $6 \times 10^8$  to  $7 \times 10^7$  copies of different HPV plasmids (listed on top) in relation to the bead-bound capture probes (listed on the left). The conjugate control (conj) serves as the positive control for correct incubation with the detection conjugate for each separate specimen. HPV2 and HPV2var (2v) are both scored as HPV2 positive.

**Clinical performance of the HSL-PCR/MPG.** The HSL-PCR/MPG yielded an HPV genotyping result identical to that of sequencing in 26 of the 45 nongenital cutaneous wart DNA samples derived from immunocompetent patients, immunosuppressed organ transplant patients, and HIV-positive patients. In 15 of 45 cases the result was compatible (i.e., an additional HPV type was detected by either method [21]) (Table 1). In four of 45 cases the results were discordant. In two of these four cases the result with one or both assays was positive for an HPV type that was not included in the other assay, and these two samples were excluded. Two other discordant results remain unresolved since repeated sequencing did not confirm the first result.

Second, the performance of the HSL-PCR/MPG assay was evaluated on cutaneous wart swabs. The positivity rate was 96% in swabs taken from nongenital cutaneous warts mainly present on hands and feet of 100 patients (see Materials and Methods). The most prevalent HPV types were HPV1 (genus mu species 1) and HPVs 2, 27, and 57 (genus alpha species 4). Together, these HPV types were detected in 89% of the cutaneous wart swabs. Other HPV types detected were HPVs 3, 4, 7, 63, 65, and 95 (Table 2). In one case the generated amplimer of the expected size, as visualized by agarose gel electrophoresis, did not hybridize with the available genotyping probes. In three other cases the HSL-PCR/MPG did not generate an amplimer of the expected size and did not give a positive signal with the genotyping assay. Within these 100 samples, 11 mul-

iple infections were observed; of these 10 contained two HPV types and 1 was positive for three types.

DISCUSSION

In this study, we have described the evaluation of a novel, sensitive, single-step broad-spectrum PCR targeting the HPV L1 region combined with a Luminex-based hybridization assay for the detection and genotyping of HPV types associated with nongenital cutaneous warts. The HSL-PCR was able to amplify

TABLE 2. HPV type distribution in 100 swabs taken from a single wart of 100 persons

Genus	Species no.	Genotype	% Positivity <sup>a</sup>
Alpha	2	HPV3	2
		HPV2	20
	4	HPV27	31
		HPV57	15
		HPV7	1
Gamma	8	HPV4	5
		HPV65	1
	1	HPV95	1
		HPV1	31
Mu	1	HPV63 <sup>b</sup>	1
	2		

<sup>a</sup> Eleven samples showed multiple infections; 10 of these samples were positive for two types and 1 was positive for three types. Four samples were negative with the genotyping assay.

<sup>b</sup> Only detected once in a multiple infection with HPV1.

HPV DNA from various sources and showed an analytical sensitivity of 1 to 10,000 HPV copies per PCR in a human genomic DNA background. The analytical HPV type specificity was 100%, as shown by analyzing amplimers generated from all 23 included HPV types at a concentration ranging from  $6 \times 10^8$  to  $7 \times 10^7$  viral genome copies per PCR. When the novel HSL-PCR/MPG method was compared with an established DNA sequencing method (13) targeting a different part of the L1 open reading frame, the results were highly concordant. Forty-one of the 43 included samples gave identical or compatible results (95%), and two gave discordant results, confirming the high analytical specificity of the novel genotyping method.

The reproducibility of the Luminex system which was used for the HSL-PCR/MPG assay has been extensively investigated by an analogue, broad-spectrum PCR genotyping assay (Digene HPV Genotyping LQ Test package insert) and was higher than 95%.

The median MFI values, representing the general background signals in all 130 analyzed clinical samples consisting of swabs and biopsy specimens, were very low, ranging from 1 to 9 MFI for the different probes. The observed cross-reactions in clinical samples were all confirmed by experiments with plasmid dilutions using an input of up to  $10^8$  viral genome copies per PCR. Furthermore, DNA sequence alignments between the probes and cross-hybridizing HPV types (data not shown) indicated that a small number of mismatches explained the cross-hybridization reactions with a maximum signal of 131 MFI. Whereas the general background signals were between 1 and 9 MFI, theoretically allowing for a low cutoff, the detected cross-hybridization reactions with some probes resulted in the use of a conventional cutoff of 200 MFI for all types. Using this high cutoff for all HPV types included in the assay could cause an underestimation of the prevalence of certain HPV types.

Amplimers of HPV27 gave cross-hybridization reactions with the probe for HPV2var that resulted in MFI signals higher than 200 MFI. The highest observed cross-reactive signal was detected in purified DNA from a cutaneous wart positive for HPV27, with an MFI value of 9,226. The cross-reactive signal for HPV2var was 534 MFI in this case. Therefore, the cutoff for HPV2var positivity was set at 700 MFI in multiple infections with HPV27 signals above 7,500 MFI.

Similarly, amplimers of HPV10 and HPV28 gave cross-hybridization reactions with the probe for HPV94 that resulted in MFI signals higher than 200 MFI, with a maximum of 221 MFI. Consequently, we used a cutoff for HPV94 positivity of 500 MFI in a multiple infection with HPV10 or HPV28 when these types had higher signals than 4,000 or 1,500 MFI, respectively.

In the absence of these types, the assay's conventional cutoff of 200 MFI was used. This cutoff value is well above the background signals and was chosen as a precaution because cutaneous warts could produce very high concentrations of viral DNA, possibly challenging the specificity of the genotyping system. The downside of this precaution is reflected by the analytical sensitivity of the assay, which ranges from 1 to 10,000 copies per PCR. However, in the panel of 100 swabs of cutaneous warts, 96 (96%) of the samples were HPV positive, indicating a high enough analytical sensitivity of the assay for this kind of sample.

Current methods for the determination the HPV genotypes

in cutaneous warts are generally based on Southern blot hybridization or on general primer-mediated PCR or type-specific PCR followed by direct DNA sequencing. Although these methods can provide accurate results, they are laborious and not suitable for use in large epidemiological studies. The assay format of the novel HSL-PCR/MPG method encompasses a broad-spectrum PCR amplifying all known cutaneous wart-associated HPV types, followed by a multiplex genotyping assay in a high-throughput setting, which allowed genotyping of a panel of PCR products derived from 96 samples in about 2 h.

The HSL-PCR was designed to amplify all HPV types previously found in cutaneous warts as well as related types from the same species (5). Complementing established types with the HPV types present in the same species is based on the assumption that these types might also be involved in cutaneous wart etiology analogous to the cervical cancer risk associated with not only HPV16 but also other types included in the same alpha-PV species 9. In this study swabs from 100 cutaneous warts were analyzed from individuals attending their general practitioners for treatment. Therefore, this panel was probably representative for warts in the general (Dutch) population. We confirmed the presence of similar HPV types as reported in the literature, but the other types present in the same HPV species were not often detected in this panel. Whether these other types occur less frequently or only in specific patient groups (e.g., HIV-infected persons) will be the subject of further investigations.

The common and easily accessible nature of cutaneous warts led early on to prolific clinical research in PV (17). Currently, these skin lesions are again the focus of attention as the clinical relevance of cutaneous warts rises with the increasing number of chronically immunosuppressed patients (9, 20). Significant physical and psychological morbidity in these patients may result from the existence of confluent plaques of warts present on cosmetically sensitive areas such as the face and hands and the presence of large warts occurring on pressure-bearing areas such as the feet. The impact of these problems and the difficulty in treating them should not be underestimated. Indeed, in some cases they may prompt the use of lower levels of immunosuppression, thus potentially endangering graft survival. In addition, the recently available preventive vaccines for HPV types causing cervical cancer pave the way for preventive and possibly therapeutic vaccines against cutaneous warts, particularly in the case of organ transplant recipients and patients with HIV. The development of these vaccines will, however, require a greater understanding of the HPV types that cause cutaneous warts and their natural history. Easy-to-use, high-throughput genotyping systems such as that described in this study will be invaluable for this future research.

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