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Evaluation of a Novel Multiplex Human Papillomavirus (HPV) Genotyping Assay for HPV Types in Skin Warts▽

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Human papillomaviruses (HPV) of the genera alpha, mu, and nu induce benign tumors of the cutaneous epithelia that constitute a significant burden for immunocompromised adults. Currently, no gold standard for genotyping of these HPV types exists. In this study, we describe the prevalence of genus alpha, mu, and nu HPV types in cutaneous warts. We developed a novel multiplex HPV genotyping assay, BSwart-PCR/MPG (BSwart), to type sensitively and specifically 19 cutaneous HPV types frequently found in warts. BSwart-PCR/MPG is based on a multiplex PCR using broad-spectrum primers and subsequent multiplex hybridization to type-specific probes coupled to Luminex beads. In a first application comprising 100 cutaneous warts, the assay was compared to another, recently described genotyping assay, the HSL-PCR/MPG. When a 10-fold dilution series was used, the detection limit was between 10 and 100 HPV genomes per PCR. When comparing the two assays, there was an excellent agreement in detecting dominant HPV types; however, we also obtained evidence for a higher sensitivity of the BSwart assay for multiple infections in these cutaneous warts. Using BSwart, HPV was found in 95% of wart preparations, with HPV1 being most prevalent, followed by types 27, 57, and 2. Both novel BSwart and HSL-PCR/MPG HPV genotyping assays are powerful high-throughput tools that could be used to learn more about the natural history of cutaneous HPV. They would be advantageous to monitor the efficacy of future skin HPV vaccines and to identify novel HPV vaccine candidates.

Human papillomaviruses (HPV) are a group of DNA viruses with more than 110 distinct genotypes characterized so far. Based on sequence relatedness, the HPV phylogenetic tree is composed of five genera (alpha, beta, gamma, mu, and nu papillomaviruses), which, in turn, are grouped into species and subdivided into types (2). Human papillomaviruses infect either cutaneous or mucosal epithelium. While the so-called high-risk HPV types within the mucosal genus alpha are associated with cancers of the uterine cervix, cutaneous HPV types of genus beta are associated with cutaneous squamous cell carcinoma (SCC) in patients with the rare hereditary skin disease epidermodysplasia verruciformis (EV) (6). Cutaneous genus alpha HPV cause benign skin lesions, such as common, flat, or plantar warts (5). These warts are common during childhood but constitute a significant burden for immunocompromised adults. For example, organ transplant recipients frequently suffer from their confluent occurrence at multiple body sites (3). As a consequence, prophylactic cutaneous genus alpha HPV vaccines are currently being developed for patients receiving organ transplantation or immunosuppression (11, 12).

Hitherto, PCR-based assays using type-specific or degenerate primers have been applied to detect and identify different cutaneous genus alpha HPV types (4). However, in order to perform HPV genotyping, these methods rely on sequencing of PCR products or cloned PCR products, which is a time-intensive procedure. To overcome the drawbacks as referred to above, a novel high-throughput multiplex broad-spectrum HPV genotyping (MPG) procedure, the HSL-PCR/MPG assay, has been described recently to genotype 23 distinct HPV types, with a type-specific analytical sensitivity of 10 to 10,000 copies per reaction (1).

At the same time, a similar Luminex-based MPG assay, the multiplex BSwart-PCR/MPG (BSwart) assay for the detection of the genus alpha, mu, and nu HPV types 1, 2, 3, 6, 7, 10, 11, 27, 28, 29, 40, 41, 43, 57, 63, 77, 91, 94, and 117, had been designed at the German Cancer Research Center. In this paper, the development of the BSwart assay and the cross-validation with the HSL-PCR/MPG assay, using 100 cutaneous wart swabs previously characterized for HPV by the HSL-PCR/MPG assay, are reported.

MATERIALS AND METHODS

Wart samples. The panel of clinical samples consisted of swabs taken from 100 cutaneous warts derived from 100 individuals (ages, >4 years) with one or more cutaneous warts who self-presented to their general practitioner (1). The swabs were taken from one wart from each patient by firmly rubbing a prewetted cotton-tipped stick 5 times over the surface of the lesion. Next, the swabs were put in 1 ml of 0.9% sodium chloride. Portions of 10 μl were used directly in the PCRs for both genotyping assays.

Plasmid clones. The analytic sensitivity and specificity of the BSwart assay were determined for each HPV type using DNA from plasmids containing the full-length HPV prototype genome after purification by the Plasmid Midi kit (Qiagen). For HPV types 91 and 94, no full-length genome was available and a fragment comprising 300 bp of the L1 gene was synthesized (Eurofins MWG Operon, Ebersberg, Germany). For determination of the analytic sensitivity, plasmid preparations were quantified using NanoDrop ND-1000 (NanoDrop

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Technologies, Wilmington, DE). Copy numbers were determined on the basis of the molecular weights of the plasmids. Endpoint dilution series (10-fold) were prepared in 100 ng/μl of human placenta DNA in a total volume of 30 μl. For colony PCR, picked DH5α bacteria, transformed by high-copy-number plasmids containing the viral genome, were used directly as a PCR template.

BSwart-PCR/MPG assay. The BSwart-PCR/MPG assay comprises the BSwart-PCR generating a biotinylated amplimer of 145 to 148 bp from the L1 region and a genotyping assay with bead-based xMAP Luminex suspension array technology, which is able to simultaneously identify 19 wart-associated HPV types from the alpha (HPV2, -3, -6, -7, -10, -11, -27, -28, -29, -40, -43, -57, -77, -91, -94, and -117), mu (HPV1 and -63), and nu (HPV41) genera. In total, 10 forward and 8 biotinylated reverse PCR primers were used as described by the manufacturer (Steinbeis Transfer Centre Multiplexion, Heidelberg, Germany). Internal DNA and PCR quality was assessed by coamplifying the human β-globin sequence (10).

Briefly, the 50-μl reactions comprised 1× Multiplex PCR kit buffer (Qiagen, Hilden, Germany), containing 3.5 mM $MgCl_2$, deoxynucleoside triphosphate (dNTP) mix, HotStartTaq DNA polymerase, 0.2 μM each HPV primer, and 10 μl of sample DNA or 1 μl of HPV plasmid dilutions. A 15-min enzyme activation step at 95°C was followed by 40 cycles of amplification in a Mastercycler (Eppendorf, Hamburg, Germany). Each cycle included a denaturation step at 94°C for 20 s, an annealing step at 38°C for 30 s, and an elongation step at 71°C for 80 s. The final elongation step was prolonged for a further 4 min. Ramping rates for the Mastercycler were adjusted as described recently (13): 1.8°C/s from 94°C to 38°C, 2.0°C/s from 38°C to 71°C, and 2.8°C/s from 71°C to 94°C. Each PCR experiment included samples with reference plasmids as positive controls and several samples lacking template DNA as negative controls.

Following PCR amplification, 10 μl of each reaction mixture was transferred to 96-well plates containing, in each well, 33 μl of tetramethylammonium chloride (TMAC) hybridization solution (0.15 mol/liter TMAC, 75 mmol/liter Tris-HCl, 6 mmol/liter EDTA, 1.5 g/liter Sarkosyl, pH 8.0), 7.0 μl of 1× Tris-EDTA (TE), and a mixture of 2,000 probe-coupled beads of each set as described recently for BSGP5+/6+-PCR/MPG, an assay for genotyping mucosal genus alpha HPV types (7–9). The mixture was heated to 95°C for 10 min, immediately placed on ice for 1 min, and then moved to a thermomixer for hybridization at 41°C for 30 min under agitation. The samples were transferred to a 96-well wash plate (Millipore, Bedford, MA), preequilibrated with washing buffer (phosphate-buffered saline [PBS], 0.02% Tween). Subsequently, the beads were washed once with 100 μl of washing buffer on a vacuum wash station (Millipore, Bedford, MA). On a horizontal shaker at room temperature, beads were resuspended for 20 min in 50 μl of streptavidin-R-phycoerythrin (Molecular Probes, Eugene, OR) diluted 1:1,600 in 2.0 mol/liter TMAC, 75 mmol/liter Tris-HCl, 6 mmol/liter EDTA, 1.5 g/liter Sarkosyl, pH 8.0. Beads were then washed three times with 100 μl washing buffer and finally resuspended in 100 μl washing buffer for 5 min on a shaker. Beads were analyzed for internal bead color and R-phycoerythrin reporter fluorescence on a Luminex 100 analyzer. The median reporter fluorescence intensity (MFI) of at least 100 beads was computed for each bead set in the sample.

BSwart cutoff definition and statistics. For each BSwart probe, MFI values from PCRs with no template (about 10% of all reactions) were considered background values. For each experiment, the cutoff was computed by adding 5 MFI to 1.2 times the median background value, as described recently (9). For all probes, this cutoff value was above the mean background plus three times the standard deviation. Due to potential cross-reactions of the HPV2 probe with HPV27 PCR products, signals of the HPV2 probe were excluded when they were lower than 12% of the HPV27 probe. HPV- and β-globin-negative samples by the BSwart assay were excluded from the analysis due to poor DNA quality. Type-specific detection differences between the two genotyping assays were assessed by chi-square tests.

HSL-PCR/MPG assay. The HSL-PCR/MPG assay was performed as described by the manufacturer (Labo Biomedical Products BV, Rijswijk, Netherlands). Briefly, it comprises a primer set with 27 nondegenerate primers (13 forward and 14 reverse) generating a biotinylated amplimer of 76 to 84 bp from the L1 region upstream of the BSwart amplification region in 35 amplification cycles.

Genotyping is performed with bead-based xMAP suspension array technology, which is able to simultaneously identify 23 wart-associated HPV types from the alpha (HPV2, -3, -7, -10, -27, -28, -29, -40, -43, -57, -77, -91, and -94), gamma (HPV4, -65, -95, -48, -50, -60, and -88), mu (HPV1 and -63), and nu (HPV41) genera (1). The assay design results in very low nonspecific background signals and therefore requires no background subtraction. The cutoffs were applied as described before (1).

TABLE 1. HPV type detection limits of BSwart-PCR/MPG with integrated β-globin detection

HPV type	Detection limit[a] in BSwart PCR
1	10
2	10
3	100
6	100
7	10
10	10
27	10
28	10
29	10
40	10
41	10
43	100
57	100
63	10
77	100
91	10
94	10

[a] Detection limit is defined as the number of plasmid copies in 100 ng human placenta DNA.

RESULTS

Analytic sensitivity and dose response. The analytic sensitivity of the multiplex HPV primers was determined for all HPV types included in the assay, except for HPV types 11 and 117, using 10-fold dilution series of plasmids containing viral L1 DNA in the presence of 100 ng/μl of human placenta DNA. BSwart detected all HPV genotypes analyzed, with an analytic sensitivity of between 100 and 10 copies of the viral genome per PCR (Table 1). Semiquantitative HPV detection was possible with various degrees of efficiency over several orders of magnitude (Fig. 1). The β-globin detection limit reached 0.1 ng of genomic DNA (17 cell equivalents). The simultaneous coamplification of β-globin did not influence the detection limit for HPV (data not shown).

Specificity. Specificity of the bead-coupled oligonucleotide probes was determined by hybridizing PCR products obtained from all 19 HPV genotypes and β-globin to a bead mixture comprising all specific probes (Table 2). To reduce the possible risk of contamination during plasmid purification, bacterial cells previously transformed with individual HPV genomes were used as a template directly (an estimated $>10^9$ plasmid copies), instead of diluted HPV plasmid DNA. Highly specific signals were observed for all 19 HPV type-specific probes with the respective PCR products (Table 2). Only the HPV2 probe weakly cross-reacted with the HPV27 PCR products.

Comparison of BSwart with HSL-PCR/MPG. In total, 8 of the 100 wart swabs were HPV negative by one of the assays (Table 3). Two samples that were positive by the HSL-PCR/MPG assays were negative for HPV and β-globin detection by the BSwart assay. Three wart swabs were found to be HPV positive only by the BSwart assay, whereas five wart swabs were HPV positive only with the HSL-PCR/MPG. Of these five, three contained gamma HPV types not included in the BSwart assay (Table 3).

Next, we compared the detection of 16 HPV types that were detected by both the BSwart and the HSL-PCR/MPG assay. The HPV prevalence determined by the two methods is presented in Table 4. Using BSwart, 95 of the 100 samples (95%)

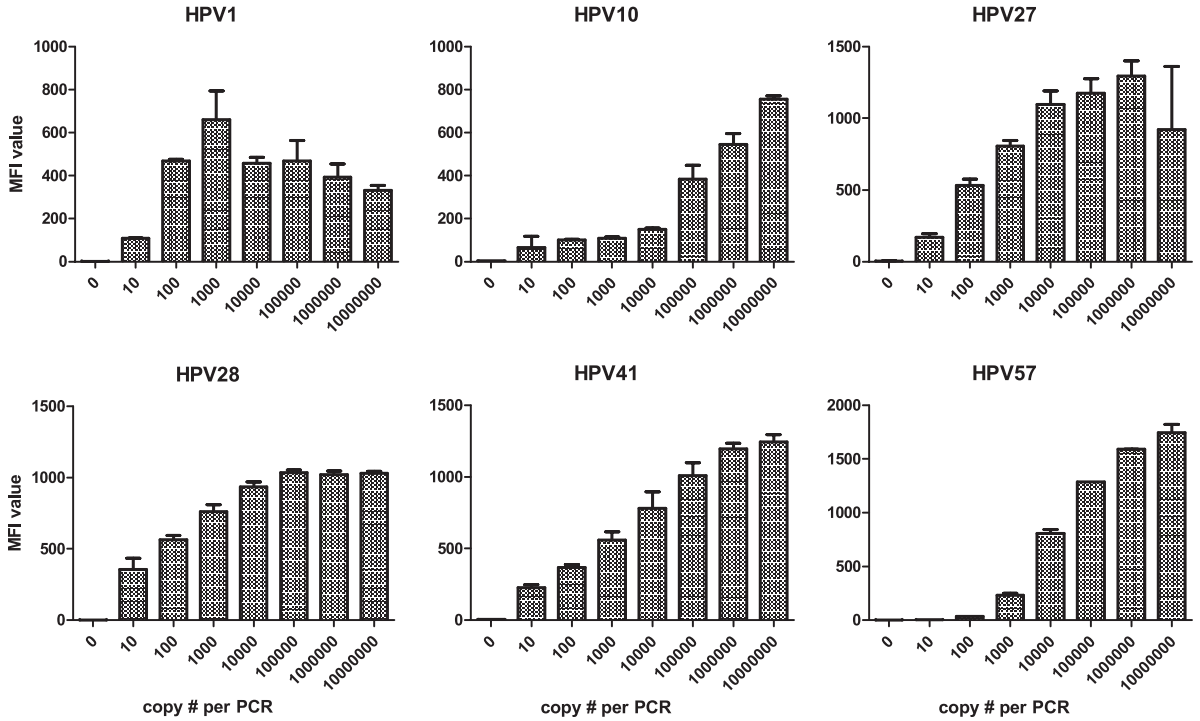

FIG. 1. Analytic sensitivity and dose response of BSwart-PCR/MPG for HPV1, -10, -27, -28, -41, and -57. Dilution series (10-fold) of plasmids containing genomic HPV DNA, diluted in 100 ng of human placenta DNA, were analyzed in duplicates by BSwart-PCR/MPG. Means and standard errors of results from two amplification and hybridization reactions are indicated.

were HPV positive and five samples were HPV negative. The most prevalent type found in the 100 warts was HPV1 (n = 40; 40.0%; genus mu, species 1) followed by HPV27 (n = 38; 38.0%), HPV57 (n = 24; 24.0%), and HPV2 (n = 22; 22.0%; genus alpha, species 4). With the HSL-PCR/MPG, 92 of the 100 samples were positive (92%); however, the result increased to 98% when HPV types of the genus gamma were also analyzed. The most prevalent HPV types were HPV1 (n = 30;

TABLE 2. Specificity of HPV PCR product detection by BSwart-PCR/MPG

HPV type of PCR product	Signal[a] detected with bead-coupled oligonucleotide probe for HPV type:																			
	1	2	3	6	7	10	11	27	28	29	40	41	43	57	63	77	91	94	117	β-globin
1	**887**	0[b]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	**1,041**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	**937**	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
6	0	0	0	**326**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	**949**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	1	**716**	0	0	0	0	0	0	0	1	0	0	0	3	0	1
11	0	0	0	0	0	0	**839**	0	0	0	0	0	0	0	0	0	0	0	0	0
27	0	19[c]	0	1	0	0	0	**743**	1	1	0	0	0	0	0	0	0	0	0	0
28	0	0	0	1	0	0	0	1	**629**	0	0	0	0	0	0	0	0	0	0	0
29	0	0	0	1	0	0	0	0	0	**1,042**	0	0	0	0	1	0	0	0	0	0
40	0	0	0	0	1	0	0	0	0	1	**564**	0	0	0	1	0	0	0	0	0
41	0	0	0	0	0	0	0	0	0	0	0	**996**	0	0	0	0	0	0	0	0
43	0	0	0	0	0	0	0	0	0	0	0	0	**452**	0	1	0	0	0	0	0
57	0	1	0	1	1	0	0	0	0	0	0	0	0	**1,018**	0	0	1	0	0	0
63	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**841**	0	0	0	0	0
77	0	0	0	1	0	1	0	0	0	0	0	0	1	1	0	**934**	1	0	0	0
91	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	**819**	0	0	0
94	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	**2,452**	0	0
117	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	**1,482**	0
β-Globin	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	**408**

[a] Specific signal (bold) is given as net median fluorescence intensity (net MFI) after the subtraction of 1.2 times the median probe-dependent background value. Cutoff was 5 net MFI.

[b] Background generated from PCR products that did not hybridize to the respective probe.

[c] Weak cross-reactivity of the HPV2 probe with HPV27 PCR product.

TABLE 3. Negative samples by either BSwart- or HSL-PCR/MPG

Sample	Result[a] or genotype(s)[b] for:		
	BSwart-PCR/MPG		HSL-PCR/MPG HPV
	β-Globin	HPV	
1	pos	**10, 28**	neg
2	pos	**94**	neg
3	pos	**1**	neg
4	neg	neg	**57**
5	pos	neg	95
6	pos	neg	65
7	pos	neg	4
8	neg	neg	**1**, 4

[a] pos, positive; neg, negative.
[b] Genotypes indicated in bold font are included in both assays.

30.0%), HPV27 ($n = 31$; 31.0%), HPV2 ($n = 20$; 20.0%), and HPV57 ($n = 15$; 15.0%).

The difference between the two assays was particularly evident for the detection of HPV types 1 ($P > 0.05$), 10 ($P < 0.05$), 27 ($P > 0.05$), 28 ($P < 0.01$), 41 ($P < 0.05$), 57 ($P > 0.05$), and 63 ($P > 0.05$) (Table 4), which were at least 22% more frequently detected by BSwart. In cases where weak signals for HPV types 1, 27, and 28 were detected only by BSwart (Fig. 2), the signal intensity corresponded to 10 to 100 HPV copy numbers per PCR compared to external standard curves (Fig. 1). However, for HPV41 (4 of 5), HPV10 (4 of 6), and HPV57 (1 of 11), additional BSwart reactions corresponded to 100 to 10,000 copy numbers per PCR (Fig. 2).

The majority of additional reactions were detected in multiple infections. As tested by HSL-PCR/MPG, 84 (91.3%) of the 92 positive wart samples for common HPV types exhibited single infections, 7 (7.6%) double infections, and 1 (1.1%) triple infection. When HPV types of the genus gamma were also included, 86 single infections (88.7%), 10 double infections (10.3%), and one triple infection (1.0%) were detected. In contrast, using BSwart, 95 warts were positive with 51 (53.7%) single, 34 (35.8%) double, 6 (6.3%) triple, 3 (3.2%) quadruple, and 1 (1.1%) sextuple infections.

Overall concordance between the two assays. The number of specimens and HPV types analyzed by both methods corresponded to a total of 1,600 signals by each method. Of these, 94 signals were concordantly positive (6.0%) and 1,438 concordantly negative (89.8%). In addition, 61 (3.8%) and 7 (0.4%) reactions additionally detected by BSwart and HSL-PCR/MPG, respectively, were discordant. This yielded a kappa value of 0.71 (95% confidence interval, 0.65 to 0.78).

Concordance in detecting dominant HPV genotypes. The intensities of MFI signals for each HPV type in a multiple infection reflect the relative concentrations of targets included in the PCR (8). For all positive reactions, positive signals were normalized to the maximum signals observed either in this study (HSL-PCR/MPG) or in the specificity experiment (BSwart). The HPV type with the highest relative value was defined as the dominant type. Among the 100 samples, 3 (3.0%) were concordantly negative, 86 (86.0%) were concordantly positive with the same dominant HPV type, and 11 (11%) were discordant. This indicated an excellent agreement between the two methods for identifying a dominant HPV type in single and multiple infections and resulted in a total concordance of 89.0%.

TABLE 4. Detection of HPV genotypes in 100 wart samples by BSwart (BS)- or HSL-PCR/MPG (HSL)

HPV type	No. of positive reactions[a]			
	BS+/HSL−	BS−/HSL+	BS+ and HSL+	BS+ and/or HSL+
1	12	3	28	43
2	4	2	18	24
3	2		2	4
7			1	1
10	6			6
27	7		31	38
28	7			7
29				0
40				0
41	5			5
43				0
57	11	2	13	26
63	5		1	6
77	1			1
91				0
94	1			1
Total	61	7	94	162

[a] Number of positive reactions for given HPV type. For example, from a total of 24 HPV2-positive samples, 18 were detected by both methods, and 4 and 2 additional reactions were detected by BSwart and HSL-PCR/MPG, respectively. HPV6, -11, and -117 were included only in the BSwart assay and showed no positive reaction. Of the HPV types included only in the HSL-PCR/MPG assay (HPV4, -48, -50, -60, -65, -88, and -95), HPV4 ($n = 5$), -65 ($n = 1$), and -95 ($n = 1$) were detected, in 4 cases as a single infection.

DISCUSSION

The novel BSwart HPV genotyping assay employed mostly broad-spectrum primers and facilitated the simultaneous and highly specific DNA detection of all 19 HPV skin wart types. The analytic sensitivity reached the level of 10 to 100 copies per PCR. The quantitative data output of the high-throughput assay avoided manual processing of the data.

Both the BSwart and HSL-PCR/MPG assay provide a so-far unique ability to genotype all genus alpha, mu, and nu cutaneous HPV types. The BSwart assay could also detect additionally the genus alpha HPV types 6, 11, and 117, while HSL-PCR/MPG identifies additionally the genus gamma HPV types 4, 48, 50, 60, 65, 88, and 95. The simultaneous detection of genus gamma HPV types by the BSwart assay was not envisioned, because we developed in parallel a novel multiplex cutaneous papillomavirus genotyping (McPG) assay at the DKFZ, which is capable of genotyping all 38 currently defined and 20 putative cutaneous HPV of the genera beta, gamma, mu, and nu (5a).

In comparison to the recently described HSL-PCR/MPG assay, the BSwart method showed a higher sensitivity in detecting various HPV types of the genus alpha, mu, and nu in plasmid dilution series as well as clinical specimens. The semiquantitative results (Fig. 1) confirmed that most of the additionally detected HPV types were present with low viral loads. As the clinical samples were preselected for HPV positivity, the increased sensitivity resulted mostly in the detection of multiple HPV types. The 35 HSL-PCR cycles (while the

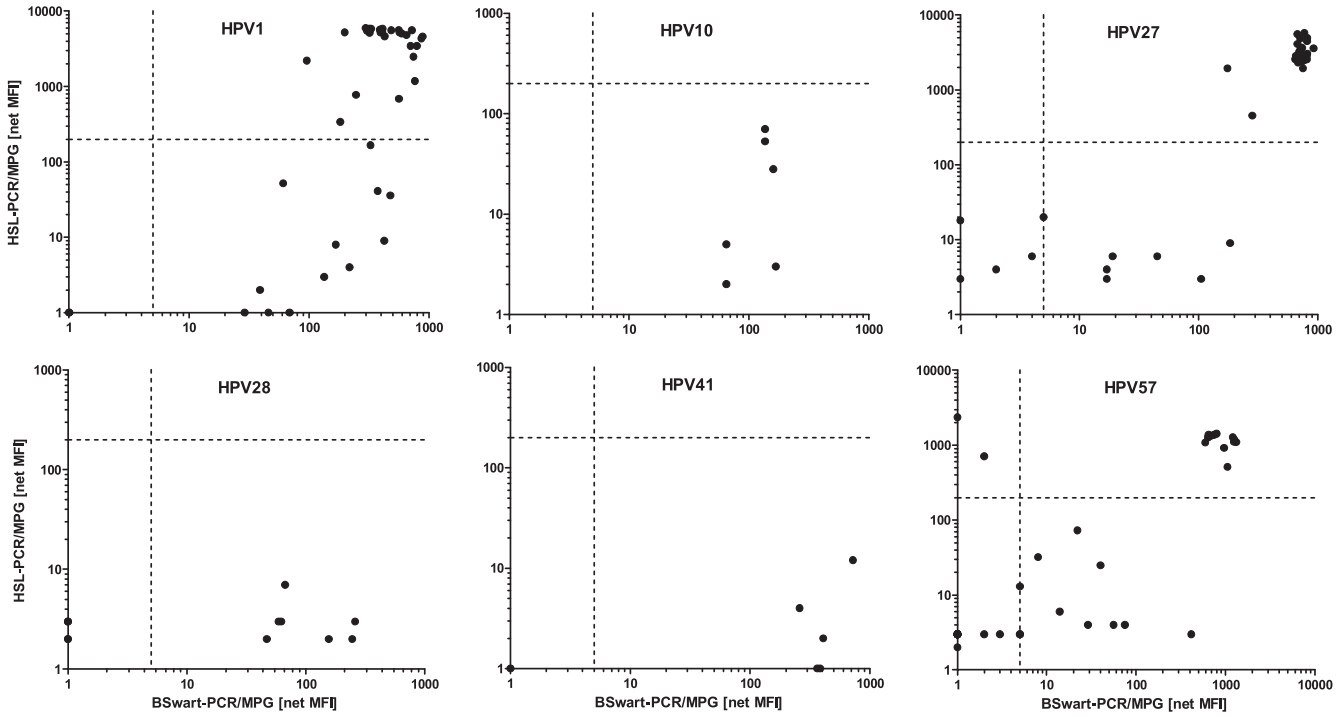

FIG. 2. Detection of HPV1, -10, -27, -28, -41, and -57 in 100 wart samples by BSwart and HSL-PCR/MPG. Net MFI values generated by the BSwart-PCR/MPG (*x* axis) are plotted against the HSL-PCR/MPG signals (*y* axis) using double logarithmic scales. The cutoffs are indicated by the dashed lines.

BSwart uses 40 cycles), the use of different PCR reagents and primers, and distinct cutoffs for interpretation of Luminex results are likely to cause the difference seen in the analytical sensitivity. By lowering the cutoff of the HSL-PCR/MPG assay, a higher concordance between the two assays for HPV types 1, 10, and 57 was observed (data not shown).

Taking advantage of the semiquantitative results of the BSwart assay, multiple HPV infections could be better characterized. Accordingly, the dominant HPV type and, thus, the genotype with the potentially highest viral load could be determined. In the vast majority of samples (89.0%), the dominant type, as determined by BSwart, was identical to the HPV type identified in the single infections by the HSL-PCR/MPG assay. Consequently, the two assays showed an excellent concordance and appeared to be able to identify the HPV type with the potentially highest viral load. It is very likely that these dominant types are also the biologically most active and, thus, the driving force in the development of skin warts.

A difference was seen in the abilities of the two assays to detect multiple HPV types in the same sample. The presence of multiple infections was surprising, as the swab specimens were taken directly from the top of warts and, thus, were expected to be positive for only one type. Nonetheless, BSwart identified a large fraction of these samples with multiple infections. The presence of different HPV genotypes in a single sample could suggest that natural competition of different HPV types may not occur on the skin. Hitherto, it was unknown whether these different viruses are present in the same cell, whether the wart is caused only by one type while additional viruses are passengers, and whether these passengers are biologically active. Viruses with apparently no detectable biological activity, however, may hint at latency in the viral life cycle consequent to evasion from the immune system. Nonetheless, by determining the dominant HPV type, the two methods agreed in 89% of cases, indicating that mostly one type is probably driving the lesion. However, in three multiple infections, two HPV types were identified by BSwart with similarly high signal intensities, suggesting that both HPV types might play a role in the development of these warts. This finding was confirmed by HSL-PCR/MPG and might be explained by the possible presence of adjacent warts caused by different HPV types in these three cases.

Identifying the individual HPV types in cutaneous warts is important for cutaneous genus alpha HPV vaccine strategies. These data are required to determine which HPV types should be included in the future vaccines and to estimate the efficacy of these vaccines in immunocompromised patients.

In conclusion, both the novel BSwart and HSL-PCR/MPG genotyping assays are powerful high-throughput tools that could be used to learn more about the natural history of genus alpha, mu, and nu skin HPV types.

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