JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2010, p. 143–149 Vol. 48, No. 1
0095-1137/10/$12.00 doi:10.1128/JCM.00991-09

Abundance of Multiple High-Risk Human Papillomavirus (HPV) Infections Found in Cervical Cells Analyzed by Use of an Ultrasensitive HPV Genotyping Assay[▽]

Markus Schmitt,[1] Bolormaa Dondog,[1,2] Tim Waterboer,[1] Michael Pawlita,[1]* Massimo Tommasino,[2] and Tarik Gheit[2]

Department of Genome Modifications and Carcinogenesis (F020), Research Program Infection and Cancer, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 242, Heidelberg 69120, Germany,[1] and Infections and Cancer Biology Group, International Agency for Research on Cancer, 150, cours Albert-Thomas, Lyon 69372, France[2]

Received 19 May 2009/Returned for modification 7 August 2009/Accepted 20 October 2009

PCR methods enable the detection of a large variety of human papillomavirus (HPV) genotypes that infect the anogenital tract. However, PCR with consensus primers, general primers, and, to a lesser extent, broad-spectrum primers may underrepresent the true prevalence of HPV, especially the true prevalence of multiple infections. We compared the rate of HPV positivity determined by a broad-spectrum PCR with primers BSGP5+ and BSGP6+ (BS-PCR) coupled to an established bead-based multiplex HPV genotyping (MPG) assay with the rate of HPV positivity determined by a multiplex PCR with type-specific primers (TS-PCR) coupled to a newly developed MPG assay for 735 selected cervical scraping samples. While the primers used for the BS-PCR are located within the L1 region of the HPV genome, the primers used for the TS-PCR target the E7 gene. The overall rates of positivity for the 19 HPV types included in both assays were 60.9% and 72.2% by the BS-PCR and the TS-PCR, respectively, and the two assays found multiple infections in 34.8% and 58.0% of the specimens, respectively. Both HPV detection assays allowed the semiquantitative detection of HPV types and identified the same dominant HPV type in 66.6% of the multiple infections. In conclusion, the TS-PCR-MPG assay significantly increased the rate of detection of HPV DNA and the number of infections with multiple HPV types detected and demonstrated that the prevalence of low-copy-number HPV infections in the anogenital tract may be strongly underestimated by conventional HPV amplification methods, especially in cases of multiple infections. As a consequence, PCR-TS-MPG appears to be highly suited for analysis of the significance of multiple infections in the development of cervical cancer and for the study the natural history and the latency of HPV.

Human papillomaviruses (HPV) are DNA viruses that infect cutaneous and mucosal epithelia. Until now, approximately 100 HPV genotypes have been fully characterized on the basis of the isolation of complete genomes (7), and there is evidence that a larger number exist (1). There are approximately 45 known mucosal HPV types; and these are further divided into three groups on the basis of their epidemiological association with cervical cancer: high-risk HPV (Hr-HPV) types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), putative high-risk HPV (pHr-HPV) types (types 26, 53, and 66), and low-risk HPV (Lr-HPV) types (e.g., types 6, 11, 40, 42, 43, 44, and 70) (18). Hr-HPV types are causally associated with several malignant diseases, of which cervical cancer has particular significance, being the second most common cancer in women worldwide and the main cancer of women in most developing countries (18). Hr-HPV type DNA has been detected in 99.7% of cervical cancer tissue specimens (26), and persistent infection with an oncogenic HPV type, particularly HPV type 16 (HPV-16) or HPV-18, is recognized as a necessary cause of cervical cancer. HPV genotyping is of importance for the study of the natural history of infections with one or several HPV types and the role of HPV persistence in the progression of cervical lesions and for the monitoring of vaccine efficacy.

Among HPV-positive women, 20 to 40% harbor in their cervices at least two types that were acquired simultaneously or successively (17). It remains controversial whether an infection with multiple types (referred to here as a multiple infection) is a risk factor for the persistence of HPV and for cervical lesions (20, 21). Moreover, it remains unknown whether women with, e.g., quadruple infections are at higher risk than women with double infections. Interest in multiple HPV infections has recently increased as prophylactic vaccines against HPV types 6, 11, 16, and 18 are expected to also provide partial protection against related HPV types by cross-neutralizing antibodies (12). Therefore, it is important to accurately type all HPV infections present in one patient. It will also be of particular interest to study the long-term impact of vaccination on the established equilibrium in the distribution of HPV types within immunized populations. Therefore, the sensitive, reliable, and unbiased profiling of the individual HPV types in patients with multiple infections is important to learn more about the natural history of HPV and to evaluate the effect of HPV vaccination.

HPV typing based on PCR methods has continuously been

* Corresponding author. Mailing address: Department of Genome Modifications and Carcinogenesis (F020), Research Program Infection and Cancer, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 242, Heidelberg 69120, Germany. Phone: 49 6221 424937. Fax: 49 6221 424932. E-mail: m.pawlita@dkfz.de.

▽ Published ahead of print on 28 October 2009.

improved over the past few years. One of the most common PCR uses the GP5+ and GP6+ primer pair, which targets conserved sequences within the L1 region of the virus genome flanking highly variable type-specific sequences (6). Use of PCR with this primer pair allows the amplification of a broad range of mucosal HPV types in a single reaction. The HPV genotype can be determined by analyzing the PCR product generated by sequence analysis, restriction fragment length polymorphism analysis, or hybridization with type-specific probes in different formats, such as the reverse line blot (RLB) assay (24) or the bead-based multiplex HPV genotyping (MPG) method (22). Recently, the performance of the PCR with primers GP5+ and GP6+ has been improved by addition of eight forward broad-spectrum primers and two backward broad-spectrum primers (primers BSGP5+ and BSGP6+ [BS-PCR]), which has led to a more homogeneous analytical sensitivity for the detection of genital HPV types (23). Consequently, the detection of HPV types 30, 39, 42, 44, 51, 52, 53, 68, 73, and 82, as well as the detection of multiple infections, was significantly improved by BSGP5+/BSGP6+ MPG. In addition, it has been shown that not only the use of degenerate primers (primers GP5+ and GP6+) (6) and/or consensus primers (primers MY09 and MY11) (2) but also the use of broad-spectrum primers (14) may lead to the underdetection of low copy numbers of HPV, particularly in cases of multiple infections (19).

Recently, a highly sensitive, multiplex type-specific HPV E7 PCR system detecting a larger proportion of multiple infections than GP5+/GP6+ RLB has been described (9). However, the detection of PCR products was based on an array primer extension (APEX) assay, a time- and labor-intensive procedure with little high-throughput ability.

In this report, we describe the development of an ultrasensitive and highly specific Luminex-based assay for the genotyping of products from the multiplex type-specific E7 PCR (TS-PCR). The results obtained by this novel high-throughput assay were compared to those obtained by the already established, sensitive BSGP5+/BSGP6+ MPG assay.

MATERIALS AND METHODS

Clinical specimens. During the course of a population-based cervical screening trial in Mongolia (8), DNA from cervical scrapings was subjected to BSGP5+/BSGP6+ PCR, followed by MPG (23). A representative set of 448 BS-PCR-positive samples and 287 BS-PCR-negative samples, among which 85 scrapings were from cervical cancer patients, were selected and reanalyzed by TS-MPG in this study.

Isolation of DNA from cervical scrapings. For the isolation of DNA from the samples from Mongolia, 2.0 ml (from a total of 20 ml) of the cervical scraping, collected with a Cervex-Brush (Rovers Medical Devices B.V., Oss, The Netherlands), in PreserveCyt solution was purified by use of a High Pure PCR template preparation kit (Roche, Basel, Switzerland), according to the manufacturer's instructions. The DNA was eluted in 0.2 ml of elution buffer (10 mM Tris, pH 8.5) and stored at −20°C until further use. The scrapings were kept for 2 months at 4°C before DNA extraction.

Type-specific E7 probe design. For each HPV type, one 18- to 21-mer oligonucleotide probe sequence (two for HPV-16) with a melting temperature of 52°C to 56°C from within the region of nonconserved sequences amplified by the type-specific E7 primers was chosen (the sequences are available upon request). All probes were tested for unspecific hybridization to biotinylated oligonucleotide primers by the use of Fast-PCR software (13) and in Luminex hybridization experiments with PCR products generated directly from freshly picked bacterial colonies harboring the genome of the respective HPV type.

Oligonucleotide synthesis. All primers and probes were purchased from Eurofins MWG Operon (Ebersberg, Germany).

Coupling of oligonucleotide probes. 5′-Amino-modifier C_{12}-linked oligonucleotide probes were coupled to distinctly colored sets of carboxylated seroMAP beads (Luminex Corp., Austin, TX) by a carbodiimide-based coupling procedure, as described previously (22).

Plasmid clones. The analytical sensitivity of the E7 type-specific primers was determined for plasmid clones of HPV types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, and 82. The DNA preparations were quantified with a spectrophotometer (Eppendorf Biophotometer, Hamburg, Germany). The copy numbers were calculated on the basis of the molecular weight of each of the plasmids. Tenfold dilution series were prepared in 100 ng/μl of human placenta (HP) DNA or HPV-negative cervical cell line C33A DNA. For evaluation of the specificity of the TS-MPG method, plasmid clones of the following HPV types were used: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, and 82. For the colony PCR, DNA from *Escherichia coli* DH5α, transformed by high-copy-number plasmids containing the viral genome, replaced the template DNA.

E7 multiplex TS-PCR. For the detection of all Hr-HPV types, all pHr-HPV types, Lr-HPV type 70, and β-globin, the PCR was performed as described previously (9), but with some modifications: a Mastercycler (Eppendorf, Germany) was used, dUTP was omitted, and reverse primers were biotinylated to allow later detection with a Luminex reader. In addition, the E7 forward primers for HPV-16 (5′-TTATGAGCAATTAAATGACAGCTCAG-3′) and HPV-39 (5′-GGTTTGCAGTTGCACACCACGG-3′) were slightly modified, resulting in a higher analytical sensitivity compared to that achieved with the previous version (4, 9).

APEX assay. The genotyping of 28 samples from Mongolia by the APEX assay was performed as described previously (9).

BSGP5+/BSGP6+ PCR. The amplification of 27 HPV types (HPV types 6, 11, 16, 18, 26, 30, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 69, 70, 73, and 82) and β-globin by BSGP5+/BSGP6+ PCR has recently been described (23).

MPG. Following PCR amplification (BS-PCR or TS-PCR), 10 μl of each reaction mixture was analyzed by MPG, as described previously (22). Briefly, the PCR products were generated, denatured, and hybridized to the bead-coupled probes in 96-well plates, which allowed the PCR products from 96 samples to be processed in parallel. After transfer of the products into wash plates with filter bottoms, the unhybridized DNA was removed. Subsequently, the biotinylated PCR products were stained with a streptavidin–R-phycoerythrin conjugate. After further washing steps, the beads were analyzed in the Luminex reader, which contains two lasers to identify the bead set by the internal bead color and to quantify the reporter fluorescence on the bead. The results are expressed as the median fluorescence intensity (MFI) of at least 100 beads per bead set.

Luminex cutoff definition and statistics. For each probe, the MFI values obtained when no PCR product was added to the hybridization mixture were considered the background values. The cutoff was computed by adding 5 MFI to 1.1× the median background value. For all probes, this cutoff value was above the mean background plus 3 times the standard deviation. The correlation between the results of the two PCR methods was assessed by the use of kappa statistics. The coefficient of variation (CV) was computed to describe the reproducibility of the assay. Type-specific detection differences were assessed by chi-square tests.

RESULTS

Probe design for TS-MPG. One β-globin-specific and 20 HPV type-specific probes for the E7 multiplex TS-PCR products were adapted from previously published APEX assay probes (9) to a final length of 18 to 21 nucleotides. However, some of these modified probes exhibited unspecific signals for hybridization to one of the biotinylated primers (data not shown), and therefore, alternative probe sequences were designed and evaluated. All novel probes were designed so that there was no mismatch to the targeted HPV prototype sequence and all other variant sequences of this type available in the NCBI nucleotide sequence database (GenBank). In addition, each probe exhibited at least three mismatches with all other mucosotropic HPV type sequences. The only exception

TABLE 1. HPV type detection limits of TS-MPG with integrated ß-globin detection

HPV type	Detection limit[a]
16	10
18	10
26	10
39	10
51	10
52	10
53	10
66	10
70	10
59	10
68	10
31	100
33	100
35	100
45	100
56	100
58	100
82	100
73	1,000

[a] The data represent the plasmid copy number in 100 ng HP DNA or C33A cell line DNA.

was the HPV-26 probe, whose sequence differed by only 1 nucleotide from the HPV-69 sequence. However, the latter HPV type was not included in our assay and the sequences of the forward and reverse PCR primers for HPV-26 E7 had three and five mismatches with the HPV-69 sequence, respectively. Therefore, the probability that HPV-69 DNA could cause a false-positive signal for HPV-26 was extremely small.

Analytical sensitivity and specificity of TS-MPG. The analytical sensitivity of the multiplex TS-PCR E7 primers with integrated β-globin primers was determined with a 10-fold dilutions series of plasmids containing genomic DNA from HPV types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, and 82 in the presence of 100 ng of human placenta DNA or HPV-negative cervical cell line C33A DNA. The PCR products were analyzed by TS-MPG. The E7 multiplex TS-PCR primer set detected all HPV genotypes analyzed, with the sensitivities ranging from 10 to 1,000 copies of the viral genome (Table 1).

The specificities of the novel E7 and the β-globin probes were determined by coupling them to defined bead sets that were subsequently hybridized with the PCR products of all 19 HPV types. No hybridization between the E7 PCR products and the β-globin probe was observed. To reduce the possible risk of contamination during plasmid purification, bacterial cells previously transformed with a plasmid containing the genome of an individual HPV type instead of purified HPV DNA were directly used as the template. The typing of all 19 HPV types was highly specific (Table 2).

Evaluation of TS-MPG with clinical samples and comparison to TS-APEX assay. We next compared the TS-MPG with the TS-APEX assay using 26 HPV DNA-positive samples (infected, on average, with four types representing the whole spectrum of HPV types analyzed) and 2 HPV-negative DNA samples from cervical cells from women from Mongolia. Of the 532 signals (28 samples × 19 HPV types) obtained by the analysis of 28 DNA samples for 19 different HPV types, 101 signals were concordantly positive (18.9%) and 413 signals were concordantly negative (77.6%). Of the 18 discordant signals, which were found only in multiple infections, 8 and 10 signals were positive by the APEX assay and MPG, respectively, yielding a kappa value of 0.89 (95% confidence interval, 0.85 to 0.94) (data not shown).

TABLE 2. Specificities of 20 type-specific probes in multiplex HPV genotyping

HPV PCR product	MFI obtained with the following HPV type-specific probe[a]:																				
	16_1	16_2	18	26	31	33	35	39	45	51	52	53	56	58	59	66	68	70	73	82	ß-Globin
16	369	289	5	10	4	16	4	13	4	15	4	9	14	9	15	10	8	15	8	6	7
18	13	2	701	11	2	9	3	12	4	12	3	9	7	5	16	6	6	15	8	6	7
26	14	2	3	152	2	10	3	13	4	12	3	8	8	5	15	6	6	23[b]	7	6	7
31	11	4	4	10	791	13	4	12	4	15	4	9	11	8	16	8	8	15	8	7	7
33	12	3	5	11	3	152	3	13	4	16	5	10	12	9	16	9	8	17	8	7	7
35	12	2	2	11	3	11	218	12	4	14	3	9	10	7	15	7	7	15	7	6	6
39	11	2	4	12	2	10	4	189	4	14	3	9	7	4	15	6	6	17	6	6	6
45	12	2	4	11	2	13	3	14	1,055	14	5	9	11	8	17	9	7	16	7	7	7
51	10	3	3	10	2	12	4	12	4	216	3	9	9	6	15	8	7	15	6	6	6
52	11	2	4	11	2	14	4	13	5	13	114	8	10	7	16	8	7	16	7	6	7
53	11	2	4	9	3	12	3	12	4	14	4	116	9	7	16	7	8	15	7	6	6
56	12	3	4	10	2	12	4	12	4	15	5	9	705	7	16	9	7	16	7	6	7
58	11	2	3	9	3	12	3	13	3	14	4	8	9	218	16	7	6	15	6	6	6
59	8	2	3	7	2	10	3	10	4	12	3	7	9	7	292	6	6	12	7	5	6
66	10	2	3	10	3	10	3	12	3	13	4	8	10	7	15	577	6	14	6	5	7
68	12	2	3	11	2	12	4	12	4	12	3	9	9	7	16	8	332	16	7	6	7
70	11	2	3	11	2	12	3	13	3	14	4	9	9	7	18	8	8	216	7	6	7
73	7	2	3	6	2	11	3	8	4	13	3	5	8	7	12	7	6	11	215	5	6
82	11	2	3	8	2	12	3	11	3	13	3	8	8	7	14	7	6	15	6	227	7
None[c]	10	2	4	10	2	10	4	13	4	13	4	8	11	9	13	10	8	13	8	7	7
Cutoff	17	7	8	17	7	18	8	18	9	20	9	15	15	13	23	13	13	22	13	12	12

[a] The data in each row represent the results for a single well in which the PCR product hybridized to a mixture of 20 distinct bead sets.
[b] The weak reactivity of the HPV-70 probe with HPV-26 was probably due to a low level of HPV contamination during PCR amplification and was not found in the clinical study.
[c] The background value is given as the MFI generated without the PCR product present.

TABLE 3. Detection of HPV genotypes in 735 clinical samples by BS-PCR or TS-PCR followed by MPG

HPV type	No. of reactions with the following result[a]:						$R_{\text{prevalence TS/BS}}$[b]	P value[c]
	BS-PCR positive, TS-PCR negative		TS-PCR positive, BS-PCR negative		BS-PCR and TS-PCR positive	BS- and/or TS-PCR positive		
	>15 MFI	<15 MFI	>15 MFI	<15 MFI				
16	21	28	16	19	118	202	0.92	0.38
18		1	17	3	37	58	1.50	0.04
26			8	1	8	17	2.13	0.07
31	1		67		36	104	2.78	<0.0001
33		1	33	32	30	96	3.06	<0.0001
35			17	1	28	46	1.64	0.03
39			21	1	22	44	2.00	0.01
45			10	1	24	35	1.46	0.14
51	10	4	47	1	42	104	1.61	<0.01
52			44	6	41	91	2.22	<0.0001
53		2	26	4	36	68	1.74	<0.01
56			15	2	25	42	1.68	0.03
58	1		9	11	34	55	1.54	0.04
59	2	4	24	1	26	57	1.59	0.03
66		3	29	11	35	78	1.97	<.001
68	1		12	6	15	34	2.06	0.01
70		1	3	5	24	33	1.28	0.34
73	1		6	11	28	46	1.55	0.06
82		6	12	1	25	44	1.23	0.39
Total	37	50	416	117	634	1,254	1.62	

[a] Number of positive reactions for the given HPV type by using a cutoff value of 5 net MFI. For example, from a total of 44 HPV-39-positive samples, 22 were detected by both methods and 1 and 21 additional reactions with values below and above a net MFI of 15, respectively, were detected by TS-PCR. HPV-69 showed no positive reaction. Net MFIs of >5 and <15 are borderline.

[b] $R_{\text{prevalence TS/BS}}$, ratio of the number of additional samples in which HPV DNA was detected by TS-PCR to the number detected by BS-PCR; e.g., HPV-53 was detected 38 times by BS-PCR and 66 times by TS-PCR, resulting in a 1.74 times more frequent detection by TS-PCR.

[c] Type-specific detection differences were assessed by the chi-square test (cutoff, >5 net MFI).

Comparison of TS-MPG with BS-MPG. We next compared the abilities of the TS-MPG and the BS-MPG, both with an integrated β-globin PCR, to detect the DNA of 19 HPV types in samples of cervical exfoliated cells from 649 women in the general population in Mongolia (8, 23) and samples from 86 Mongolian cervical cancer patients. The prevalence of HPV in the 735 samples determined by the two methods is presented in Table 3. The number of specimens and HPV types analyzed corresponds to a total of 13,965 signals by each method. Of these, 626 signals were concordantly positive (4.5%) and 12,724 signals were concordantly negative (91.1%). In addition, 615 signals were discordant (4.4%), yielding a kappa value of 0.65 (95% confidence interval, 0.62 to 0.68). The overall prevalence of HPV was higher by TS-MPG (72.2%) than by BS-MPG (60.9%).

Of the discordant signals, TS-MPG failed to detect 82 infections but identified 533 additional infections compared to the number detected by BS-MPG (Table 3). For HPV-16, TS-MPG detected 35 additional reactions and BS-MPG detected 49 additional reactions ($P = 0.38$), both with mostly borderline signals (<15 net MFI), indicating that the samples had very low viral loads. This finding was independent of the different TS-MPG HPV-16 probes, as both probes always gave identical results. Nevertheless, for most Hr-HPV types, the exceptions being HPV types 16, 26, 45, 70, 73, and 82, TS-MPG was significantly more sensitive (Table 3), especially in cases of multiple infections (see the next paragraph). The difference between the two assays was particularly evident for the detection of HPV types 26, 31, 33, 39, 52, and 68, for which the detection rates by TS-MPG were increased two- to threefold compared to the detection rates of BS-MPG (Table 3). The remaining Hr-HPV types were 1.2- to 2-fold more frequently detected by TS-MPG than by BS-MPG. Of the 287 samples negative for 1 of the 19 HPV types by BS-MPG, TS-MPG identified at least 1 HPV type in 106 cases (36.9%). Among those, 79 (27.5%) were single infections, 19 (6.6%) were double infections, 7 (2.4%) were triple infections, and 1 (0.4%) was a quadruple infection.

Among the 292 samples showing single infections by BS-MPG (Table 4), 23 were negative by TS-MPG (7.9%), 134 (45.9%) showed single infections, and 135 (46.2%) showed multiple infections. Summing up all positive findings for these 292 samples, TS-MPG detected 466 HPV infections and, thus, found 1.6-fold more HPV infections than BS-MPG (292 types). In addition, BS-MPG detected 416 HPV infections among the 156 samples

TABLE 4. Number of HPV types found in 735 clinical samples by BS-PCR and TS-PCR

PCR type	No. of samples with the following no. of HPV types[a]:									
	0	1	2	3	4	5	6	7	8	9
TS	204	223	151	82	37	11	12	9	4	2
BS	287	292	96	33	16	5	6			

[a] Number of samples obtained by using a cutoff of 5 net MFI. 0, HPV negative; 1 to 9, infection with a single HPV type to infection with nine HPV types, respectively.

with multiple infections, while TS-MPG found 551 infections, resulting in a 1.3-fold difference.

The majority of the additional infections detected by TS-MPG were found in samples with multiple infections, resulting in the designation of 58.0% of the samples as having multiple infections (308 of a total of 531 samples HPV positive by TS-MPG; Table 4). Up to nine HPV types were detected in a single sample by TS-MPG. Multiple infections (156 of 448 samples HPV positive by BS-MPG) were identified by BS-MPG in 34.8% of the samples.

Reproducibility of TS-MPG for detection of multiple infections. We analyzed the reproducibility of the TS-PCR for 19 HPV types by retesting 60 clinical specimens infected with 4 to 9 HPV types 30 months after the first analysis. By using a cutoff of 5 net MFI, 255 of the total of 1,140 signal pairs were concordantly positive (22.4%) and 814 were concordantly negative (71.4%). Seventy-one signals were discordant (6.2%), yielding a kappa value of 0.84 (95% confidence interval, 0.80 to 0.87). Fourteen of the discordant pairs were positive only in the second analysis, while 57 were positive only in the first analysis, probably due to repeated thawing and freezing of the samples. When borderline-positive reactions (cutoff, <15 net MFI) were excluded, the kappa value improved to 0.88 (95% confidence interval, 0.84 to 0.90). For the detection of HPV-16, four of the five discordant pairs showed borderline reactions, leading to kappa values of 0.82 (95% confidence interval, 0.67 to 0.97) and 0.96 (95% confidence interval, 0.89 to 1.0) by the use of cutoffs of 5 and 15 net MFI, respectively. Thus, despite the long-term storage and the repeated use of specimens, the reproducibility of TS-MPG for the genotyping of HPV in samples infected with four or more types appears to be very high.

Detection of dominant HPV genotypes. The intensities of the MFI signals for each HPV type in a multiple infection reflect the relative concentrations of the targets included in the PCR (23). For all positive samples, positive reactions were normalized to the maximum signal observed in this study for the particular type by the particular method, and the HPV type with the highest relative value was defined as the dominant type. Among the 425 samples showing one dominant Hr-HPV type by both methods, a total of 283 (66.6%) were concordantly positive for the same dominant HPV type, while 142 (33.4%) were discordant, indicating a good agreement between the two methods for the identification of a dominant HPV type in single and multiple infections. Among the 142 samples in which HPV-16 was the dominant type by TS-MPG and/or BS-MPG, HPV-16 was concordantly dominant in 73 (51.4%) by both methods, 51 (25.9%) by BS-MPG only, and 18 (12.7%) by TS-MPG only.

DISCUSSION

We describe here a novel combination of assays consisting of an ultrasensitive multiplex type-specific PCR for HPV E7 for the detection of mucosal Hr-HPV types in combination with a highly specific and sensitive multiplex HPV genotyping assay. This assay showed a very high degree of reproducibility for the detection of multiple infections and even higher rates of detection of overall HPV positivity and multiple infections in comparison to the already established high sensitivity of the PCR-MPG with broad-spectrum BSGP5+/PCR-MPG (23).

The addition of primers BSGP5+ and BSGP6+ to the GP5+/GP6+ PCR reduced the number of mismatches with poorly detectable HPV types and reduced the competition for the amplification of the different HPV types present in the same sample. Despite the coamplification of β-globin, the BS-PCR proved to be more sensitive than its "gold standard," GP5+/GP6+ PCR. In the present work, however, many HPV infections, mostly multiple infections, were still missed. This is possibly due to the competition for the amplification of related HPV types. This problem, common to all broad-spectrum primer-mediated PCR methods, occurs if, for example, one HPV type is present in great excess over another type (23, 25). The competition for HPV types by primers during PCR could be drastically reduced by the use of HPV type-specific primers. Indeed, the TS-PCR targeting the E7 gene in combination with the APEX assay, a chip-based HPV detection system, recently showed a high degree of sensitivity for the detection of multiple infections (9). Here, we showed that the results of TS-MPG assay demonstrated very good agreement with the results of the APEX assay, while the MPG assay appeared to be slightly more sensitive. In contrast to the APEX assay, however, the MPG assay allowed a much faster, cheaper, and less laborious means of genotyping for epidemiological studies. In total, up to nine HPV types could easily be detected in a single reaction. The use of type-specific primers minimized the risk of amplification bias because of infection with a dominant HPV type. Therefore, type-specific primers will be the better tool for future studies analyzing whether infection with multiple Hr-HPV types is a risk factor for the development of cervical cancer.

These data suggest that the overall HPV prevalence of 46.0% in the Mongolian population determined by BSGP5+/BSGP6+ MPG (23) may be substantially higher by an ultrasensitive method, such as the TS-PCR-MPG assay. This disparity highlights the fact that HPV infection rates in specific settings vary considerably according to the HPV detection methods used.

The increased sensitivity of the TS-PCR-MPG assay can be explained by the use of HPV type-specific primers rather than by the probes used in the detection system (MPG). While they are restricted in the number of HPV types that they detect, the TS primers not only reduced the number of mismatches to specific HPV types but also minimized the competition for the amplification of HPV DNA from multiple infections. To exclude the possibility of cross-hybridization as a reason for the detection of additional HPV types, cross-reactivities with bacterial colony PCR products were examined for all 19 HPV types. These highly concentrated PCR products, originating from millions of HPV copies, did not show cross-reactivity in a single instance, thus demonstrating the excellent specificity of the TS-PCR-MPG assay.

Moreover, BS-PCR can simultaneously amplify more than 50 distinct HPV types (M. Schmitt et al., unpublished data). Even if a type is not detected by 1 of the 27 type-specific probes included in the assay, the coamplification of the undetected HPV types directly competes with the amplification of the Hr-HPV types, explaining the reduced sensitivity for the detection of multiple infections. Taking the 8 Lr-HPV types in-

cluded in the BS-PCR assay into account, a total of 125 additional Lr-HPV types were detected, increasing the proportion of multiple infections to 37.2% (184 of 495) (data not shown).

An additional factor is the buffer and polymerase composition of the TS-PCR assay mixture. By reanalyzing some samples by BS-PCR with the same PCR kit from Qiagen, the MFI signals and detection rates increased (data not shown). This effect was substantial but still too small to account for the entire increase in the sensitivity of the TS-PCR assay.

To date, 20 to 40% of HPV-positive women have been reported to be infected with multiple HPV types (3, 10, 17, 23); however, technical differences as well as study design differences hamper a direct comparison of these results. Coinfections were found more frequently among young women and among those with cytological abnormalities or an impaired immune response (5, 11, 16). Higher proportions of populations at high risk of cervical cancer tended to harbor multiple HPV types than populations not belonging to the risk groups mentioned above. In the present study, a surprisingly high proportion of multiple infections (58%) were detected among HPV-positive women by the TS-PCR-MPG assay. It is worth mentioning that the TS-PCR-MPG assay analyzed only 19 Hr- and pHr-HPV types. This implies that the actual number of coinfections may be even higher when Lr-HPV types are also taken into account. It further shows that the real prevalence of multiple infections has been largely underestimated in the past. As such, it has been reported that by combining data obtained by five different HPV genotyping methods, more than 93% of multiple infections were double or triple infections (15). However, we showed, using a single but ultrasensitive assay, that more than 25% of multiple infections contained four or more HPV types. This may be important, especially for the investigation of potential cross talk between HPV types in the induction and progression of cervical lesions and for the analysis of viral latency or clearance and type-specific immunity against certain types after previous exposure.

The great variety of different HPV genotypes present in a single sample suggests that natural competition among different HPV types may not occur in the uterine cervix. It remains the subject of speculation whether the presence of one HPV type will increase the likelihood of acquiring a second HPV type. In addition, it is completely unknown whether these viruses are present in the same cell and whether they are biologically active, and the viral loads are also unknown. 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, the distribution of genital HPV types appears to resemble the situation known for skin HPV types, in which coinfections with more than 10 genotypes present at very low copy numbers are frequently detected (1).

It is worth mentioning that analysis for the dominant HPV type and, thus, the genotype with the potentially highest viral load by both methods identified a high percentage of concordant results (66.6%). If it is assumed that the dominant types identified by the BS-PCR are correct, as the type with the highest viral load should outcompete the other types, the TS-PCR-MPG correctly identified a surprisingly high proportion of dominant types. The remaining discordances between both methods may be attributed to (i) missing or minimal competition in the TS-PCR, (ii) additional amplification of potentially dominant Lr-HPV types by BS-PCR, and (iii) differences in type-specific detection limits between the two methods. Further studies are imperative to verify whether these dominant types are also the biologically most active and, thus, the driving force in the development of cervical lesions.

In conclusion, the results of the present investigation provide further evidence for the notion that cervical HPV infection, in particular, infection with multiple types at low copy numbers, is more widespread than was previously suggested and was overlooked in the past due to methodological issues. Predominantly in an era in which HPV vaccination commences, an improved understanding of the natural history and dynamics of HPV infection and the pathogenic effects of multiple types is necessary in order to monitor the impact of vaccination on changes in the distribution of individual HPV types. The technology will be suited for the analysis of large sample collections to address the question of whether multiple infections are associated with an increased risk for the development of cervical cancer.

ACKNOWLEDGMENTS

The study was partially supported by grants from La Ligue Contre le Cancer (Comité du Rhône, Drôme and Savoie), the Association pour la Recherche sur le Cancer, the European Union (LSHC-2005-018704), the Association for International Cancer Research (to M.T.), and BMBF/HGF-Cancéropôle Grand-Est. M.S. received a Ph.D. grant from the DKFZ and the Studienstiftung des Deutschen Volkes.

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