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Modified General Primer PCR System for Sensitive Detection of Multiple Types of Oncogenic Human Papillomavirus▽

Anna Söderlund-Strand,[1,2] Joyce Carlson,[3] and Joakim Dillner[1,2]*

WHO HPV LabNet Global Reference Laboratory, Department of Clinical Microbiology,[1] and Department of Laboratory Medicine,[2] Lund University, Malmö University Hospital, SE-20502 Malmö, Sweden, and Department of Clinical Chemistry and Pharmacology, Lund University Hospital, SE-22185 Lund, Sweden[3]

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Human papillomavirus (HPV) infection is a necessary cause of cervical cancer and cervical dysplasia. Accurate and sensitive genotyping of multiple oncogenic HPVs is essential for a multitude of both clinical and research uses. We developed a modified general primer (MGP) PCR system with five forward and five reverse consensus primers. The MGP system was compared to the classical HPV general primer system GP5+/6+ using a proficiency panel with HPV plasmid dilutions as well as cervical samples from 592 women with low-grade cytological abnormalities. The reference method (GP5+/6+) had the desirable high sensitivity (five copies/PCR) for five oncogenic HPV types (HPV type 16 [HPV-16], HPV-18, HPV-56, HPV-59, and HPV-66). The MGP system was able to detect all 14 oncogenic HPV types at five copies/PCR. In the clinical samples, the MGP system detected a significantly higher proportion of women with more than two concomitant HPV infections than did the GP5+/6+ system (102/592 women compared to 42/592 women). MGP detected a significantly greater number of infections with HPV-16, -18, -31, -33, -35, -39, -42, -43, -45, -51, -52, -56, -58, and -70 than did GP5+/6+. In summary, the MGP system primers allow a more sensitive amplification of most of the HPV types that are established as oncogenic and had an improved ability to detect multiple concomitant HPV infections.

Infection with certain types of human papillomavirus (HPV) is a necessary risk factor for cervical cancer (2). In particular, type-specific persistence of HPV increases the risk for malignant transformation (11, 15, 17). HPV genotyping is important for most of the major medical indications of HPV testing. For example, genotyping of HPV has been shown to be an accurate indicator of treatment failure in follow-up after treatment for cervical dysplasia (1, 22). In cervical screening, HPV genotyping better identifies women at high risk of dysplasia than does a nongenotyping HPV test (6), and monitoring of the types that are associated with the highest risk for cancer is particularly important (3, 22). The monitoring of the effect of HPV vaccination requires adequate HPV genotyping to evaluate whether vaccine HPV types disappear from the population and how the prevalence of nonvaccine types is affected (8). Although the clinical validity of HPV genotyping is well documented, no HPV genotyping test has yet been approved by the U.S. Food and Drug Administration, and no optimal algorithm for risk assessment based on genotyping results in the clinical management of HPV infections has yet been accepted (4).

The most widely used HPV genotyping methods are PCR based, using either a series of type-specific PCRs or general primer PCRs followed by a genotyping test. Common general primers, all targeting the well-conserved L 1 gene of the HPV genome, include the GP5+/6+ primer pair with a limited number of mismatches against target templates (7), the MY 09/11 primers containing degenerate nucleotides (21), and sets of several forward and reverse primers such as the SPF10 primers, which also have some nucleotides replaced with inosine for detection of additional HPV types (12), and the PGMY 09/11 primers (10). PCR products can be genotyped by hybridization to HPV plasmids (9) or to genotype-specific oligonucleotides. Available commercial methods include the Roche Linear Array HPV genotyping test (Roche, Germany), which allows for detection of 37 genital HPV types, and the Inno-LiPA assay (Microgen Bioproducts, United Kingdom), with the capacity to detect 25 genital HPV types. Genotyping with a higher throughput can be obtained using fluorescent beads on the Luminex xMAP platform (Luminex Corporation, Texas), which performs genotyping in a 96-well format (18).

We have developed a new primer system in which the classical GP5+/6+ general primers have been modified for improved amplification of 14 oncogenic genital HPVs, with one-step multiplex high-throughput genotyping using the Luminex system. The modified general primer (MGP) system was evaluated for its sensitivity and specificity for individual HPV types by using a proficiency panel of HPV plasmids in serial dilutions, both alone and in combination. Cervical samples from 592 patients with atypical smears were then analyzed using both GP5+/6+ primers and MGPs, and the results are compared.

MATERIALS AND METHODS

Plasmid controls. Plasmids with type-specific inserts of the high-risk (HR) HPV type 16 (HPV-16), -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68 and of the low-risk HPV-6, -11, -42, -43, -70, -72, and -82 were used for assessment of sensitivity and specificity of the GP5+/6+ and the MGP PCR

* Corresponding author. Mailing address: WHO HPV LabNet Global Reference Laboratory, Department of Clinical Microbiology, Lund University, Malmö University Hospital, SE-20502 Malmö, Sweden. Phone: 46 40 338126. Fax: 46 40 337312. E-mail: joakim.dillner@med.lu.se.

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TABLE 1. MGP sequences and the original GP5+/6+ sequences[a]

Primer	Former name[b]	Direction	Sequence, 5′–3′
GP5+		Forward	TTTGTTACTGTGGTAGATACTAC
MGPA	FA	Forward	**ACGTTGGATG**TTTGTTACTGTGGT**G**GATACTAC
MGPB	FB	Forward	**ACGTTGGATG**TTTGTTAC**C**GT**T**GT**T**GATACTAC
MGPC	New	Forward	**ACGTTGGATG**TTTGTTACT**AA**GGTAGATAC**C**AC**TC**
MGPD	FD	Forward	**ACGTTGGATG**TTTGTTACTGT**T**GT**G**GATAC**A**AC
MGP31	New	Forward	**ACGTTGGATG**TTTGTTACT**A**TGGTAGATAC**C**AC**AC**
GP6+		Reverse	GAAAAATAAACTGTAAATCATATTC
MGPG	RG	Reverse	**ACGTTGGATG**GAAAAATAAACTGTAAATCATATTC**CT**
MGPH	RH	Reverse	**ACGTTGGATG**GAAAAATAAA**T**TGTAAATCATA**C**TC
MGPI	RI	Reverse	**ACGTTGGATG**GAAATATAAA**T**TGTAAATCA**A**ATTC
MGPJ	RJ	Reverse	**ACGTTGGATG**GAAAAATAAACTGTAAATCATATTC
MGP18	New	Reverse	**ACGTTGGATG**GAAAAATAAACTG**C**AAATCATATTC

[a] Nucleotides that differ from the GP5+/6+ primers are in bold. All reverse primers are biotinylated at the 5′ end.
[b] For primers described by Söderlund-Strand et al. (20).

systems. The DNA concentration of all plasmids diluted in Tris-EDTA (TE) was determined using the PicoGreen double-stranded DNA quantitation reagent kit (Molecular Probes, Oregon). Pools of plasmids of all 14 HR HPV types diluted in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) with a background of human DNA (10 ng/μl of human DNA; Sigma-Aldrich, Sweden) to two concentrations, 100 copies/μl and 1 copy/μl before PCR amplification, were used as positive controls in each run. The pools were designed to avoid cross-hybridization between probes and amplicons within the pool and contained (i) HPV-16, -18, and -51; (ii) HPV-31, -33, and -58; (iii) HPV-39, -45, -52, and -56; and (iv) HPV-35, -59, -66, and -68.

An HPV DNA proficiency panel produced by the WHO HPV LabNet (www.who.int) for validation of HPV genotyping methods was also analyzed with both primer systems. The panel consisted of pooled HPV plasmids of HPV-6, -11, -16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68 at concentrations of 100 and 10 copies of each type/μl (and for HPV-16 and -18 also one copy [international unit]/μl) diluted in TE with human placental DNA. The HPV proficiency panel was pooled to contain (i) HPV-16, -45, -52, and -33; (ii) HPV-11, -18, -31, and -51; (iii) HPV-35, -39, -59, and -66; and (iv) HPV-6, -56, -58, and -68.

Study population. The samples that were used for validation of the modified PCR primers are derived from a randomized health care policy that evaluates the impact of HPV testing in triaging among women with the cytological diagnoses of atypical squamous cells of undetermined significance (ASCUS) or cervical intraepithelial neoplasia grade I (CIN I) in Stockholm, Sweden. In the study, 3,319 women with unclear or low-grade smears on screening were randomized into two arms with different follow-up strategies. The first arm used colposcopy of all women. In the second arm the Hybrid Capture II HPV test was used for triaging and only women positive for HPV were referred for colposcopy. The present evaluation of methods utilizes samples selected from among the 1,600 women in the HPV testing arm. We included all 329 cases with a histopathology-verified diagnosis of CIN II or worse (CIN II+) and 263 samples selected at random from the 1,271 women without CIN II+ in histopathology (mild dysplasia, atypia, or benign diagnosis) (total, 592 samples). Of CIN II+ cases 97.9% were HPV positive with Hybrid Capture II, and 62.0% of patients with diagnoses of lower severity were HPV positive with this test. As Hybrid Capture II was used for referral, current evaluation of sensitivity of two PCR tests cannot use comparison to the Hybrid Capture II data, only comparison of the tests to each other.

DNA preparation. Cervical cells were obtained using the Hybrid Capture DNA specimen collection kit (Digene, Maryland) and stored in specimen transport medium (Digene) at −20°C until extraction using sodium dodecyl sulfate and proteinase K (16). The extracted DNA was diluted in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored at −20°C until analysis.

Analysis with GP5+/6+ primers. GP5+/6+ consensus primers (7) were used to amplify HPV DNA in a 25-μl PCR mixture containing 0.5 μM of each primer, 200 μM of each deoxynucleoside triphosphate, 1× PCR buffer II (Roche, Germany), 3.5 mM $MgCl_2$, 0.2% bovine serum albumin, 0.65 units AmpliTaq Gold DNA polymerase, and 5 μl sample. The reverse primer was biotinylated at the 5′ end. Nontemplate controls contained sterile water instead of DNA as template in the reaction mixture. The denaturing step was performed at 94°C for 10 min followed by 45 cycles of 94°C for 1.5 min, 50°C for 2 s, 40°C for 1.5 min (7% ramp, 50°C to 40°C [0.2°C/s]), and 72°C for 2 min. In the present work, all PCR amplifications were performed in a Mastercycler (Eppendorf, Denmark) thermocycler.

The cutoff using the GP5+/6+ primers was set to twice the mean fluorescence intensity (MFI) of the nontemplate controls with a minimum MFI cutoff of 10, and adjustments were made for cross-hybridization between the following: (i) the probe for HPV-16 with HPV-31, -45, -56, -59, and -66 amplicons; (ii) the probe for HPV-52 with HPV-66 amplicons; (iii) the probe for HPV-58 with HPV-56 amplicons; (iv) the probe for HPV-59 with HPV-18, -33, and -39 amplicons; and (v) the probe for HPV-68 with HPV-31 amplicons. If the MFI for the amplicon genotype was >100, the MFI of the cross-hybridizing probe was considered positive if it was more than twice the cutoff for that probe. In HPV-33-positive samples, HPV-59 was considered positive if its signal was greater than that for HPV-33.

Design and optimization of new primers. The general primers GP5+/6+ (7) were modified for improved annealing to 14 HR HPV types (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68) using Oligo 6.0 software (Cambio Ltd., United Kingdom) resulting in five forward and five reverse primers, called MGPs, which each have a few modified nucleotides for better annealing to a group of HPV types (Table 1). The number of mismatches was minimized, and A-C mismatches were avoided. No degenerate nucleotide sites were used, in order to minimize variation between primer batches. Each primer has a 5′ 10-nucleotide extension for improved thermodynamic stability. The initial design and evaluation of some of these primers included detection of their amplification products using HPV genotype-specific mass extend primers in the SEQUENOM system (20). To further improve the amplification of HPV-18 and -31, one forward primer has been altered and one new forward primer and one new reverse primer have been added compared to our previous publication ("new" in Table 1). All reverse primers were biotinylated at the 5′ end.

MGP PCR. The MGP PCR program was optimized and evaluated by agarose gel electrophoresis. Each PCR mix of 25 μl contained 0.3 μM of each primer, 200 μM of each deoxynucleoside triphosphate, 1× PCR buffer II (Roche), 2.5 mM $MgCl_2$, 0.5 units AmpliTaq Gold DNA polymerase, and 5 μl sample. PCR amplification was performed with denaturation at 95°C for 10 min followed by five cycles of 95°C for 30 s, 42°C for 30 s, and 72°C for 45 s and then 45 cycles of 95°C for 30 s, 64°C for 30 s, and 72°C for 45 s, with a final step at 72°C for 10 min.

Cutoff limits and cross-hybridization. For determination of cutoff limits and to exclude false-positive results due to cross-hybridization, serial 10-fold dilutions of single HPV plasmids of the 21 HPV types were used from 500,000 copies to 5 copies/PCR. Individual cutoff levels for each probe were established using the MFI of the signals for nontemplate controls plus five times the standard deviation of these MFIs with the exception of HPV-51, -59, -66, and -68, for which cutoffs were three times the MFI of the nontemplate controls. The minimum cutoff was an MFI of 5.

Cross-hybridization was observed as follows: (i) the probe for HPV-16 with HPV-31, -56, and -66 amplicons; (ii) the probe for HPV-52 with HPV-66 amplicons; (iii) the probe for HPV-58 with HPV-56 amplicons; and (iv) the probe for HPV-59 with HPV-18, -33, and -39 amplicons. The cutoff level required to entirely eliminate false positives due to cross-reactivity for each of these probe/amplicon pairs was defined as the mean of all ratios derived from the signal of the cross-reacting probe divided by the signal of the true amplicon-matching probe

[i.e., (MFI cross-reacting probe)/(MFI true amplicon-matching probe)] for each single plasmid amplicon plus five times the standard deviation of this ratio.

HPV DNA detection system. Detection of biotinylated HPV amplicons in 10-μl aliquots of the PCR mix was achieved by hybridization to short oligonucleotide probes covalently linked to fluorescence-labeled carboxy-coated polystyrene beads on the Bioplex 200 Luminex system (Bio-Rad, California). The coupling of probes to beads was performed as previously described (18), with some modifications. In short, 12.5 million carboxylated beads were suspended in 125 μl of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid, pH 4.5 (MES), after which 2 nmol of probes and 1 mg of *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC) were added, and the mix was incubated for 30 min under agitation in the dark. The addition of EDC and the incubation were repeated, and the beads were washed first with 1 ml of 0.2 g/liter Tween 20 and then with 1 ml of 1 g/liter sodium dodecyl sulfate before storage in 200 μl of TE buffer at 4°C. The type-specific probe sequences previously described (18) were used for HPV DNA detection of HPV-6, -11, -16, -18, -31, -33, -35, -39, -42, -43, -45, -51, -52, -56, -58, -59, -66, -68, -70, -73, and -82. The probe for HPV-68 was altered to 5′ amino-modifier C_{12}-GCT GTG TAT GAT TCT AAT AAA T 3′ (M. Schmitt, personal communication), and a probe for an HPV-35 variant (35v) common in Sweden (5′ amino-modifier C_{12}-CTG CTG TGT CTA CTA GTG A 3′, in house) and a second universal probe (19) were added. The same set of 24 probes (22 HPV-type-specific probes and two universal probes) and the exact same coupling batches were used with both primer systems for comparison. The hybridization of probes to PCR products was performed according to the method of Schmitt et al. (18). Results were recorded as MFI in the Luminex system. Signals are reported as the ratio of MFI for the sample to the cutoff MFI, i.e., the signal/cutoff ratio.

Statistical analysis. To determine whether one of the tests consistently gave stronger detection signals than the other, the signal-to-cutoff ratios obtained with the two methods were compared using the Wilcoxon signed-rank test (SPSS software; SPSS Inc., Illinois).

RESULTS

Detection limit. The results of the HPV proficiency panel for 500 and 50 copies of PCR template for 14 HR types and HPV-6 and -11 as well as five copies of HPV-16 and -18 showed that all HPV types could be detected at 50 copies with GP5+/6+ primers, except for HPV-31 and -58, which were detectable at 500 copies, and HPV-6, -35v, -39, -51, -52, and -68, which were not detectable. With MGPs all 16 HPV types could be detected at the lowest copy number tested (Table 2).

Positive controls consisting of 14 pooled HR types further diluted to five copies of plasmids/PCR showed that GP5+/6+ detected HPV-16, -18, -56, -59, and -66 at five copies/PCR and that MGP detected all 14 HR types at five copies/PCR (Table 3).

Comparison of GP5+/6+ and MGP systems was also performed using 329 cervical samples from women with uncertain or low-grade cytological abnormalities who were found to have an underlying CIN II+ lesion in histopathology and 263 samples from women with uncertain or low-grade cytological abnormalities who did not have CIN II+ in histopathology. Mean signal-to-cutoff ratios for all concordant positive samples are shown in Table 3. Signal-to-cutoff ratios obtained with MGPs for HPV-11, -16, -33, -35v, -43, -51, -52, -68, and -70 were on average more than twice as high as those obtained with GP5+/6+.

There were also 13 discrepant results that were GP^+/MGP^- and 240 results that were GP^-/MGP^+ (Table 4). All discrepant results for HPV-11, -18, -33, -35, -35v, -39, -42, -43, -45, -52, -58, -59, and -70 were positive only with MGP. Among the discrepant results, HPV-56 had a positive signal that was at least twice the background, i.e., a signal-to-cutoff ratio of ≥2, with GP5+/6+. For this type, there was only one discrepant GP5+/6+-positive result, but the signal/cutoff ratio was very

TABLE 2. Mean signal/cutoff ratios of three analyses of the pooled HPV DNA proficiency panel using GP5+/6+ and MGP[a]

HPV type and copy no.	PCR system			
	GP5+/6+		MGP	
	Mean (SD) signal/cutoff ratio	Mean cutoff	Mean (SD) signal/cutoff ratio	Mean cutoff
HPV-6		46		43
500	0.83 (0.11)		3.2 (0.5)	
50	0.54 (0.19)		1.6 (0.4)	
HPV-11		35		24
500	4.1 (1.9)		10.0 (2.4)	
50	2.1 (0.5)		6.1 (1.6)	
HPV-16		20		16
500 (alone)	51.2 (13.0)		62.7 (6.4)	
500	38.6 (2.7)		49.7 (5.8)	
50 (alone)	33.0 (14.1)		47.1 (8.1)	
50	24.0 (3.0)		37.1 (7.0)	
5 (alone)	4.7 (3.0)		12.5 (10.5)	
HPV-18		13		9
500 (alone)	17.9 (5.1)		22.5 (4.5)	
500	9.1 (2.9)		24.1 (7.1)	
50 (alone)	5.9 (2.8)		11.6 (4.8)	
50	3.8 (2.0)		12.0 (6.5)	
5 (alone)	3.7 (3.4)		6.8 (3.0)	
HPV-31		10		5
500	3.5 (2.5)		14.7 (4.1)	
50	0.8 (01)		5.0 (0.6)	
HPV-33		10		5
500	3.0 (0.6)		5.6 (1.9)	
50	1.1 (0.2)		3.2 (0.9)	
HPV-35		14		12
500	1.7 (0.5)		6.3 (1.2)	
50	1.0 (0.2)		2.2 (0.8)	
HPV-35v		10		5
500	0.4 (0.0)		3.2 (3.1)	
50	0.3 (0.1)		1.6 (1.6)	
HPV-39		24		17
500	0.9 (0.1)		8.6 (0.1)	
50	0.6 (0.0)		2.7 (0.5)	
HPV-45		10		6
500	5.6 (1.9)		14.2 (6.9)	
50	2.8 (1.1)		15.4 (7.7)	
HPV-51		10		10
500	0.9 (0.5)		12.7 (3.2)	
50	0.4 (0.0)		6.7 (2.2)	
HPV-52		13		9
500	0.9 (0.0)		118.6 (19.2)	
50	0.5 (0.0)		96.0 (7.8)	
HPV-56		10		5
500	24.9 (1.5)		92.2 (9.9)	
50	4.7 (1.0)		39.9 (17.9)	
HPV-58		10		6
500	2.9 (0.1)		4.7 (0.7)	
50	0.5 (0.1)		1.8 (1.1)	
HPV-59		12		16
500	22.7 (2.2)		26.4 (7.5)	
50	11.0 (5.4)		8.0 (4.2)	
HPV-66		11		17
500	27.3 (7.2)		33.4 (2.9)	
50	14.4 (1.4)		11.7 (2.3)	
HPV-68		10		9
500	0.4 (0.1)		5.4 (0.4)	
50	0.4 (0.1)		1.7 (0.3)	

[a] HPV-16 and -18 were also analyzed alone. 35v is a variant of HPV-35.

TABLE 3. Comparison of GP5+/6+ PCR and MGP PCR systems for plasmid controls and for all concordant (i.e., positive by both GP5+/6+ and MGP PCRs) clinical samples[a]

HPV type	GP5+/6+ PCR system						MGP PCR system						No. of concordant positive samples
	Mean ratio for no. of copies:		Mean cutoff	MFI of positive samples (median)	Mean ratio of samples		Mean ratio for no. of copies:		Mean cutoff	MFI of positive samples (median)	Mean ratio of samples		
	500	5			Positive	Negative	500	5			Positive	Negative	
16	34.9	18.6	17	557 (587)	32.8	0.67	67.2	43.0	14	991 (1,017)	73.4	0.69	191
18	15.8	3.3	11	130 (129)	11.8	0.50	20.5	10.3	10	153 (130)	16.5	0.63	62
31	6.1	0.5	10	99 (91)	9.9	0.22	26.3	3.8	5	95 (103)	19.1	0.43	66
33	4.1	0.6	10	53 (51)	5.3	0.20	16.8	4.8	5	57 (38)	11.4	0.41	44
35	1.5	0.7	16	19 (19)	1.1	0.52	3.5	2.3	13	29 (29)	2.0	0.64	2
35v	0.6	0.3	10	31 (28)	3.1	0.21	2.3	1.4	5	45 (42)	9.1	0.40	18
39	3.4	0.7	20	75 (71)	3.9	0.52	8.5	5.0	16	57 (52)	3.6	0.66	16
45	6.8	0.7	10	95 (86)	9.5	0.46	16.6	3.3	7	101 (85)	13.8	0.63	34
51	2.9	0.44	10	56 (55)	5.6	0.30	26.1	13.5	10	115 (110)	12.0	0.36	29
52	4.1	0.6	11	179 (106)	15.4	0.50	172.3	108.5	8	1147 (1,118)	149.0	0.66	35
56	54.2	3.8	10	374 (440)	37.4	0.21	118.8	68.4	5	357 (371)	71.3	0.41	46
58	3.9	0.5	10	31 (32)	3.1	0.24	7.1	1.7	5	43 (37)	5.3	0.49	14
59	43.8	19.4	10	286 (323)	28.9	0.47	36.3	14.1	14	350 (147)	26.8	0.36	21
66	18.3	3.6	10	352 (274)	35.0	0.57	36.3	11.4	12	411 (411)	29.1	0.34	40
68	0.5	0.4	10	10 (10)	1.0	0.40	5.7	3.4	12	39 (39)	3.9	0.34	1
6[b]	ND	ND	35	76 (63)	2.7	0.53	ND	ND	62	101 (84)	2.0	0.48	13
11[b]	ND	ND	35	123 (141)	3.5	0.50	ND	ND	21	191 (226)	9.0	0.85	6
42[b]	ND	ND	10	116 (105)	11.6	0.33	ND	ND	6	82 (63)	14.5	0.57	28
43[b]	ND	ND	10	30 (30)	3.0	0.29	ND	ND	6	46 (46)	9.2	0.53	1
70[b]	ND	ND	10	96 (83)	9.6	0.29	ND	ND	5	126 (121)	23.1	0.54	16
73[b]	ND	ND	50	74 (79)	1.6	0.48	ND	ND	44	87 (94)	2.2	0.67	5
82[b]	ND	ND	246	529 (573)	2.7	0.49	ND	ND	275	532 (514)	2.2	0.61	4

[a] Ratio denotes sample signal/cutoff. ND, not done.
[b] These types were not included in the positive controls.

high (88.1). DNA sequencing of this sample showed a variant of HPV-56 with 96% similarity to the prototype HPV-56 sequence. At the primer sites of this HPV-56 variant, one more mismatch was found between template and MGP than between template and GP. All other discrepant samples that were positive for a type with GP5+/6+ that was not detected with MGP were positive for other HR types with MGP.

For MGP^+/GP^- samples, mean signal/cutoff ratios of ≥ 2 were found for HPV-6, -11, -16, -31, -35, -39, -42, -45, -51, -52, -56, -58, -59, -66, -68, and -70. Significantly more samples were positive for HPV-16, -18, -31, -33, -35v, -39, -42, -43, -45, -51, -52, -56, -58, and -70 using the MGP than using the GP5+/6+ system (Table 4).

One woman was HPV positive with only GP5+/6+, and 27 women were HPV positive with only MGP (Table 4). The one woman who was HPV positive with only GP5+/6+ had CIN I histopathology, whereas of the 27 women HPV positive with only MGP one had CIN I histopathology, eight had CIN II or worse (CIN II+) histopathology, three had CIN I cytology, and 15 had normal or low-grade abnormalities in histopathology or cytology. Among all women with CIN II+ in histopathology, 93.6% were HR HPV positive with the GP5+/6+ system and 96.4% were HR HPV positive with the MGP system. The HR HPV positivity among women with ASCUS/CIN I in cytology but who did not have CIN II+ in histopathology was 60.1% with GP5+/6+ and 66.9% with MGP.

Thirty-five women had three HPV types, six had four types, and one woman had five types detected with GP5+/6+, whereas 65 women had three types, 18 had four types, and 19 had five (or more) types detected with MGP.

DISCUSSION

We have developed a new primer system that, together with an altered PCR program, has a sensitivity that is superior or equivalent to that of the classical GP5+/6+ primer system. This is shown both by detection of lower numbers of plasmid copies and by an increased number of type-specific positive results. More multiple infections were also detected by the new MGP than by the GP5+/6+ system. Furthermore, the mean ratio of signal to cutoff for concordant positive results for HPV-11, -16, -33, -35, -43, -51, -52, -68, and -70 increased more than twofold with MGP amplicons compared to GP5+/6+ amplicons, implying better sensitivity and presumably a more accurate interpretation of the test results. According to the analysis of the discrepant results, MGP detected significantly more infections for 14 out of 21 tested types, whereas GP5+/6+ did not detect any HPV type significantly better than did MGP.

In the 592 clinical samples, 227 more HPV-positive results were detected with MGP than with GP5+/6+. Even though both GP5+/6+ and MGP detected the last dilution in the plasmid titrations for HPV-16, -18, -56, -59, and -66 (five copies/PCR), the MGP system identified many more samples having these types than the GP5+/6+ system did. It is possible that the MGP system may have even higher sensitivity than the last plasmid titration tested or that it may be more robust in resistance to inhibition or competition from multiple HPV infections in clinical samples. Twenty-seven women were HPV positive only after MGP PCR, compared to a single woman, bearing an HPV-56 variant, who was positive only after

TABLE 4. Mean signal/cutoff ratios for clinical samples with discordant results between the GP5+/6+ and the MGP PCR systems[f]

HPV type	No. of GP^+/MGP^- samples	Mean ratio for GP^+ samples	Histopathology/ cytology result for GP^+/MGP^- samples	No. of GP^-/MGP^+ samples	Mean ratio for MGP^+ samples	Histopathology/cytology result(s) for GP^-/MGP^+ samples	Significance[a]	Total no. of discordant results
16	2 (0[g])	1.1	NA	12 (2[b,g])	6.2 (14.5[h])	Condyloma,[b] benign	$P < 0.005$	14
18	0	0	NA	10 (0)	1.5	NA	$P < 0.05$	10
31	1 (0)	1.3	NA	17 (1)	3.4 (18.8)	CIN III	$P < 0.05$	18
33	0	0	NA	6 (0)	1.9	NA	$P < 0.05$	6
35	0	0	NA	2 (2[c])	2.8 (2.2)	Two CIN II[c]	NS	2
35v	0	0	NA	5 (1[c])	1.6 (1.6)	CIN II[c]	$P < 0.05$	5
39	0	0	NA	14 (2[c])	2.9 (4.2)	Benign, CIN II[c]	$P < 0.005$	14
45	0	0	NA	16 (1)	4.6 (8.4)	Benign	$P < 0.0005$	16
51	1 (0)	1.0	NA	31 (7[b,d])	8.5 (11.6)	Benign, condyloma,[b] atypia, CIN I,[d] CIN II, CIN NOS, CIN III	$P < 0.0005$	32
52	0	0	NA	47 (3[e])	51.2 (115)	ASCUS, CIN I, cancer[e]	$P < 0.0005$	47
56	1 (1)	88.1 (88.1[h])	CINI	18 (4)	3.7 (4.7)	Two benign, condyloma, CIN I	$P < 0.005$	19
58	0	0	NA	16 (1[e])	3.6 (6.7)	Cancer[e]	$P < 0.0005$	16
59	0	0	NA	1 (0)	3.14	NA	NS	1
66	1 (0)	1.2	NA	3 (0)	2.4	NA	NS	4
68	3 (0)	1.3	NA	3 (1)	4.8 (8.3)	Benign	NS	6
6	1 (0)	1.3	NA	4 (0)	2.2	NA	NS	5
11	0	0	NA	1 (0)	2.0	NA	NS	1
42	0	0	NA	15 (2[d])	2.4 (1.7)	Benign, CIN I[d]	$P < 0.005$	15
43	0	0	NA	6 (2)	1.6 (1.3)	Benign, ASCUS	$P < 0.05$	6
70	0	0	NA	9 (1)	8.4 (1.2)	CIN I	$P < 0.05$	9
73	2 (0)	1.2	NA	1 (1)	1.0 (1.0)	Benign	NS	3
82	1 (0)	1.2	NA	3 (1)	1.7 (2.0)	CIN III	NS	4
Total	13 (1)	NA	NA	240 (27)	NA	NA	NA	253

[a] Wilcoxon signed-rank test for analysis of signal ratios. NS, $P > 0.05$.
[b] One sample is MGP positive for the HPV-16 and -51 probes.
[c] One sample is MGP positive for the HPV-35, -35v, and -39 probes.
[d] One sample is MGP positive for the HPV-42 and -51 probes.
[e] One sample is MGP positive for the HPV-52 and -58 probes.
[f] NA, not applicable; CIN NOS, CIN, no other specification.
[g] Values in parentheses in this column are the numbers of samples that were negative for all HPV types by the other method.
[h] Values in parentheses in this column are the means of signal/cutoff ratios for samples that were negative for all HPV types by the other method.

GP5+/6+ PCR. Among women with CIN II+ histopathology, GP5+/6+ missed eight women, of whom one had cancer, and MGP missed none. MGP detected a higher number of HR HPV-positive samples than did GP5+/6+ both among women with and among women without CIN II+. The high HPV prevalence among women with CIN I or less is not surprising since the samples were obtained from women who had had ASCUS/CIN I in cytology.

The MGP PCR system differs from the classical GP5+/6+ in both primer design and PCR strategy. Whereas the GP5+/6+ PCR utilizes 45 identical cycles, each with an initial 1.5-min. denaturation followed by a 50°C to 40°C annealing ramp, the MGP system has significantly shortened the denaturation step to 30 s, has provided five initial cycles with a permissive annealing temperature of 42°C, and has then introduced stringent brief annealing at 64°C for the remaining 45 cycles. We believe that shorter denaturation and more stringent annealing contribute to more efficient amplification in the MGP PCR but presumably also explain why MGP PCR missed the HPV-56 variant, with an extra mismatch in the primer region for MGP.

Whereas the classical GP5+/6+ PCR system has been in use for more than 10 years for detection of a large number of HPV types, the full range of HPV types detectable with MGP primers has not yet been explored.

The use of type-specific probes for detection limits the possibility of detecting variants having sequence variation at the site of the probe. This is exemplified by the fact that two separate HPV-35 probes (35 and 35v) were needed to detect all HPV-35-positive cases. Since the probes do not hybridize at the primer sites, there is no difference between GP5+/6+ and MGP in this aspect. The use of "universal" probes that will detect any HPV type present in addition to the type-specific probes can potentially identify new types that can then be identified by sequence analysis.

It can be noted that the MGP principle of cutoff determination could not be applied to the GP5+/6+ system, since the latter system had too-low variability in the nontemplate controls, which made it impossible to make a proper distinction between true positives and background. Therefore, for each system the cutoff principle which had been optimized for that particular system was used, resulting in somewhat different cutoff levels.

Systems using multiple primers are usually better for detect-

ing multiple concomitant infections than are systems based on a single primer pair (5). This is in accordance with our finding that the MGPs could detect more than twice the number of multiple infections, 102 samples compared to 42 detected using the GP5+/6+ primers.

It has been shown that HPV-31 is an HR type that is particularly difficult to detect at low copy numbers (13, 14). When designing the MGPs, we made a particular effort to ensure improved amplification of this particular type, giving a significantly improved MFI-to-cutoff ratio for HPV-31.

Some of the most commonly used primer systems for amplification of HPV are the MY 09/11 primers (21); the modified version of these, the PGMY 09/11 primers (10); and the SPF primers (12). Instead of using degenerate nucleotides (as in MY 09/11), which leads to a certain batch-to-batch variation, or inosine (as in SPF), which does not hybridize well with any base, we designed the MGPs according to the principle of choosing the mismatch with as low an adverse effect on hybridization as possible and avoiding all mismatches with a strong repelling effect.

A recent study that compared different genotyping methods on the same set of samples found quite different results for the different methods (13). This is in accordance with the findings of the present study where some HPV types were detected in much higher numbers with MGP than with GP5+/6+, most distinctly HPV-51 and -52. The difference is sufficiently large to be predicted to lead to considerably different results in surveys of HPV prevalence at the population level. It is essential that HPV genotyping assays that are used for epidemiological studies define the sensitivity for different HPV types (alone or in combination) in a comprehensive and standardized manner.

In summary, we have modified the single primer pair GP5+/6+ into a multiprimer system and adjusted PCR parameters with improved amplification of at least 14 HPV types, among those the most important carcinogenic types, HPV-16 and -18. The modified primer system also detected more multiple infections. Taken together, the MGP PCR system could be useful in epidemiological research, for primary HPV screening, for HPV triaging, for follow-up after treatment for cervical dysplasia, and for monitoring of HPV prevalence in the current era of HPV vaccinations.

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