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Updating the PGMY Primers and Probes for Improved Detection of HPV68a: Validation of Version 2 of the PGMY-CHUV Assay

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HPV68a is not efficiently detected by PCR with the PGMY primers. Version 2 of the PGMY-CHUV assay (PGv2) was developed from version 1 (PGv1) to evaluate HPV68-discordant results with the Anyplex II HPV28 assay. We now report that PGv2 is significantly more sensitive than PGv1 for HPV68a and as sensitive and specific for the other HPV genotypes during a 1-year prospective validation ($n = 714$ samples).

Cervical cancer is caused by long-term persistent infections with high-risk (HR) anogenital human papillomaviruses (HPV) genotypes (1, 2). HPV16 and -18 account for 70% of the cervical cancer cases worldwide, and most of the remaining cases are associated with other HR genotypes (HPV31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, -68, -73, and -82) (1, 3). Among them, HPV68 is divided into two subtypes, "a" and "b" (ME180 cell line). During our evaluation of the Anyplex II HPV28 kit (Seegene, Seoul, South Korea), nearly half of the cases positive for HPV68 were undetected by version 1 of the PGMY-CHUV assay (PGv1) (4). This was the anticipated consequence of the inefficient amplification of HPV68a using assays based on the well-established PGMY primer set (5). PGv1 is similar to the widely used Linear Array (LA) assay (Roche). Both rely on multiplex PCR targeting the L1 open reading frame with biotinylated PGMY primers, followed by reverse blotting hybridization (RBH) of the biotinylated amplicons against a panel of HPV genotype-specific probes immobilized on a membrane. Hybrids are then revealed with a peroxidase-based reaction leaving a colored precipitate for LA or a chemiluminescent signal recorded on a film for PGv1 (6–9). Many laboratories throughout the world use LA, and several reference laboratories within the WHO HPV Laboratory Network (LabNet) use PGv1 (5). PGv1 and LA have similar performances for HPV genotyping overall and for HPV68 in particular (6). HPV68 prevalence therefore is underestimated by laboratories relying on either LA or PGv1. While HPV68 presently accounts for a low proportion of cervical cancer cases, its prevalence may increase after the implementation of the nonavalent vaccine (i.e., that including HPV6, -11, -16, -18, -31, -33, -45, -52, and -58), which does not contain HPV68 (10).

To improve HPV68 coverage, we updated PGv1 with the RSMY09-L primer and the HPV68a probe specific to version 2 (PGv2), both published in Estrade and Sahli (4). This allowed us to resolve the discordant samples that were positive for HPV68 with the Anyplex II HPV28 kit and that were negative with PGv1 (4). The additional RSMY09-L primer and the HPV68a-specific probe may alter the sensitivity and specificity of PGv2 toward other HPV genotypes. For this reason, and to confirm its performance for HPV68a, we prospectively evaluated PGv2 against PGv1 on all samples submitted to our laboratory during 1 year ($n = 762$ specimens, of which cervical smears, $n = 531$; paraffin-embedded tissue and biopsies, $n = 123$; other smears, $n = 108$). PCR and genotyping were performed with one 50-μl PCR mixture containing 3 mM $MgCl_2$ for each PGv1 or PGv2, the appropriate primer mixture (PGv1 or PGv2), and 5 μl DNA, as described previously (6). After PCR, each reaction was evaluated by gel electrophoresis and subjected to RBH if it was doubtful (smeary DNA profile, very weak HPV amplicon near 450 bp) or positive (distinct amplicon at 450 bp). The PCR-negative samples were not subjected to RBH and recorded as HPV negative in our database. The samples that were negative for the human gene internal control and for HPV DNA were considered inadequate and excluded from the analysis ($n = 48$ samples [6.3%]). DNA sequencing using the PGMY09 or the PGMY11 primer set was used to resolve PCR-positive samples that were negative or weakly positive after RBH, according to Estrade et al. (6). Sequencing with the RSMY09-L primer was also performed for the HPV68a-positive samples. Statistical analyses for the 32 genotypes represented on the probe array (HPV6, -11, -16, -18, -26, -31, -33, -34, -35, -39, -40, -42, -44, -45, -51, -52, -53, -54, -55, -56, -57, -58, -59, -66, -68a, -68b, -69, -70, -73, -82, -83, and -84) were performed as described previously (4, 6). The genotypes that were not represented on the membrane array and found by sequencing only were not statistically evaluated, owing to their low prevalence ($n = 34$ total [1 HPV30, 5 HPV61, 7 HPV62, 3 HPV67, 4 HPV72, 2 HPV74, 3 HPV81, 5 HPV89, 2 HPV90, and 2 untypeable]).

The genotyping results of the 714 informative samples (high-grade cases, $n = 48$; atypical squamous cells of undetermined significance cases, $n = 273$; low-grade cases, $n = 170$; and unknown or follow-up cases, $n = 223$) are shown in Table 1. The agreement interpretation was perfect for HPV11, -31, -33, -34, -54, -55, -66, -68b, and -83 ($\kappa = 1$), near perfect for HPV6, -16, -39, -42, -44, -45, -51, -52, -53, -56, -58, -59, -70, -73, -82, and -84 ($0.839 < \kappa < 0.977$), strong for HPV18 ($\kappa = 0.797$), and poor for HPV68a ($\kappa = 0.141$). The agreement interpretation after the kappa (κ) statistics was omitted for HPV26 ($n = 2$), -34 ($n = 1$), -40 ($n = 2$), -57 ($n =$

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TABLE 1 Genotype-specific comparison of PGv1 and PGv2[a]

Genotype[b]	Risk[c]	No. of samples for each result[d]:				% agreement	Total no. positive	% positive agreement	Kappa data			Two-tailed McNemar's *P* value
		−/−	+/−	−/+	+/+				κ	SD	Int.[f]	
6	L	672	2	3	37	99.30	42	88.1	0.933	0.030	NP	1.000
11	L	705	0	0	9	100.00	9	100.0	1.000	0.000	PE	1.000
16	H	657	2	3	52	99.30	57	91.2	0.950	0.022	NP	1.000
18	H	702	0	4	8	99.44	12	66.7	0.797	0.099	ST	0.125
26	L	712	0	0	2	100.00	2	100.0	1.000	0.000	NA	1.000
31	H	696	0	0	18	100.00	18	100.0	1.000	0.000	PE	1.000
33	H	707	0	0	7	100.00	7	100.0	1.000	0.000	PE	1.000
34	L	713	0	0	1	100.00	1	100.0	1.000	0.000	NA	1.000
35	H	709	0	0	5	100.00	5	100.0	1.000	0.000	PE	1.000
39	H	692	0	1	21	99.86	22	95.5	0.976	0.024	NP	1.000
40	L	712	0	1	1	99.86	2	50.0	0.666	0.315	NA	1.000
42	L	677	1	4	32	99.30	37	86.5	0.924	0.034	NP	0.375
44	L	700	1	0	13	99.86	14	92.9	0.962	0.038	NP	1.000
45	H	699	1	0	14	99.86	15	93.3	0.965	0.035	NP	1.000
51	H	679	2	0	33	99.72	35	94.3	0.969	0.022	NP	0.500
52	H	685	1	1	27	99.72	29	93.1	0.963	0.026	NP	1.000
53	L	675	7	2	30	98.74	39	76.9	0.863	0.045	NP	0.180
54	L	706	0	0	8	100.00	8	100.0	1.000	0.000	PE	1.000
55	L	710	0	0	4	100.00	4	100.0	1.000	0.000	PE	1.000
56	H	696	1	2	15	99.58	18	83.3	0.907	0.053	NP	1.000
57	L	713	0	0	1	100.00	1	100.0	1.000	0.000	NA	1.000
58	H	687	3	0	24	99.58	27	88.9	0.939	0.035	NP	0.250
59	H	694	0	1	19	99.86	20	95.0	0.974	0.026	NP	1.000
66	H	685	0	0	29	100.00	29	100.0	1.000	0.000	PE	1.000
68a	H	701	0	12	1	98.32	13	7.7	0.141	0.126	po	0.000[e]
68b	H	702	0	0	12	100.00	12	100.0	1.000	0.000	PE	1.000
69	H	714	0	0	0	100.00	0	NA	NA	NA	NA	NA
70	L	703	0	3	8	99.58	11	72.7	0.840	0.091	NP	0.250
73	H	702	0	2	10	99.72	12	83.3	0.908	0.065	NP	0.500
82	H	708	1	0	5	99.86	6	83.3	0.908	0.091	NP	1.000
83	L	709	0	0	5	100.00	5	100.0	1.000	0.000	PE	1.000
84	L	699	1	0	14	99.86	15	93.3	0.965	0.035	NP	1.000
Total, including HPV68a		22,321	23	39	465	99.73	527	88.2	0.936	0.007	NP	0.057
Total, excluding HPV68a		21,620	23	27	464	99.77	514	90.3	0.945	0.007	NP	0.671

[a] PGv1, PGMY-CHUV version 1 (standard PGMY primer set); PGv2, PGMY-CHUV version 2 (equivalent to PGv1 with the additional RSMY09-L primer and HPV68a-specific probe).
[b] Only the 32 genotypes represented on the array were considered for analysis.
[c] L, low risk; H, high risk. The classification of the risk group was according to Estrade et al. (6). HPV26 was classified as low risk for the sake of simplicity, although it may be a high-risk or risk-undetermined genotype.
[d] −/−, negative with both assays; +/−, PGv1 positive and PGv2 negative; −/+, PGv1 negative and PGv2 positive; +/+, positive with both assays.
[e] Interpretation (Int.) of the κ values. PO, poor; ST, strong; NP, near perfect; PE, perfect; NA, not applicable.
[f] $P < 0.05$, two-tailed McNemar's test.

1), and -69 ($n = 0$), since they were present at a very low frequency.

The discordant cases were distributed equally between PGv1 and PGv2 ($P > 0.125$ by two-tailed McNemar's test), except for HPV68a, which was significantly more efficiently detected by PGv2, as expected ($P = 0.000$ by two-tailed McNemar's test). The 13 HPV68a-positive samples corresponded to 4 single infections and 9 multiple infections with up to 3 additional HPV genotypes ($n = 5$ samples with single additional infection by HPV16, -31, -42, -58, or -62; $n = 3$ samples with double additional infections by HPV42 and -53, HPV35 and -66, and HPV53 and -66; $n = 1$ sample with triple additional infection by HPV39, -53, and -58). PGMY amplicon sequencing of these 13 HPV68a-positive samples identified the HPV68a subtype in the 4 single infections and in the 4 multiple infections in which the HPV68a hybridization signals were strong (data not shown), hence confirming the specificity of the HPV68a probe (4). DNA sequencing otherwise identified the major HPV genotype found in each of the remaining 5 multiple infections, as expected.

The other discordant cases were significantly associated with low viral loads overall ($P < 0.0001$ by the chi-square test for trend; Table 2). Only the genotypes having a sufficient number of discordant cases were individually examined and reported in Table 2. Except HPV68a, all showed a significant trend for discordance at low viral loads. We and others have shown that viruses present at low viral loads are overrepresented in discordant cases, independently of the method used (4, 6, 11). This can be explained by the stochastic amplification of viral DNA at low concentrations.

TABLE 2 Distribution of HPV6, -16, -18, -42, -53, and -68a discordant and concordant results, according to viral load

Genotype	Risk[a]	No. of samples by viral load[b]								P^c
		+−		+		++		+++		
		Disc.	Conc.	Disc.	Conc.	Disc.	Conc.	Disc.	Conc.	
6	L	3	1	2	3	0	10	0	23	<0.0001
16	H	3	1	2	7	0	30	0	14	<0.0001
18	H	3	1	1	3	0	4	0	0	0.0244
42	L	3	3	2	9	0	14	0	6	0.0038
53	L	7	0	2	1	0	8	0	21	<0.0001
68a	H	2	0	1	0	3	1	6	0	0.3481[d]
Total, including HPV68a		38	31	13	95	5	213	6	126	<0.0001
Total, excluding HPV68a		36	31	12	95	2	212	0	126	<0.0001

[a] L, low risk, H, high risk.
[b] +−, doubtful PCR and barely detectable RBH signal; +, weak; ++, medium; +++, strong PCR/RBH signal (6). The numbers indicated are pooled from single and multiple infections. Only the specific genotypes with ≥4 discordant results are shown. Disc., number of discordant cases; Conc., number of concordant cases. The attribution to the viral load category was based on the higher value for each pair (PGv1 versus PGv2 result).
[c] Chi-square analysis for trend addressing whether discordance is associated with viral load.
[d] $P > 0.05$.

With PGv1 as a reference (excluding HPV68a), the sensitivity and specificity of PGv2 for HPV overall were 95.3% (464/487) and 99.9% (21,620/21,647), respectively. With PGv2 as a reference (including HPV68a), the sensitivity and specificity of PGv1 for HPV overall were 92.3% (465/504) and 99.9% (22,321/22,344), respectively (Table 1). Therefore, PGv2 is more sensitive for HPV68a than PGv1, with comparable sensitivity and specificity for the other genotypes. These results confirm the successful evaluation of PGv2 with the 2013 WHO quality control (data not shown).

Studies using genotyping methods relying on the original PGMY primers, such as the Linear Array (Roche) or PGMY-CHUV version 1, may underestimate the prevalence of HPV68 in patient populations similar to ours by a factor of 2, as suggested by the relative yearly occurrences of both subtypes in our population (HPV68a, $n = 13$; HPV68b, $n = 12$; Table 1). HPV68a and HPV68b accounted for 25 occurrences in total, which would place HPV68 at the 6th position according to the number of occurrences, between HPV58 and HPV39 in the high-risk group. If the nonavalent vaccine successfully reduces the prevalence of its target genotypes, HPV68 would rank third after HPV51 and -66 among the high-risk genotypes represented in our patient population. In this situation, the HPV genotyping assays ought to target the two subtypes of HPV68 in order to efficiently detect a clinically relevant proportion of cervical lesions in the future (12, 13).

In conclusion, this updated PGMY primer and probe set will be useful for the comprehensive epidemiological assessment of cervical cancer and high-grade cases that will arise in spite of vaccination, knowing that HPV68 is neither included in the presently used vaccines nor in the foreseen nonavalent vaccine (10).

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