

Human Papillomavirus Quantification in Urine and Cervical Samples by Using the Mx4000 and LightCycler General Real-Time PCR Systems[∇]

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During the last decade, growing efforts have focused on human papillomavirus (HPV) detection using liquid hybridization, conventional PCR, and real-time PCR-based methods to increase the overall proportion of patients participating in cervical cancer screening procedures. We proposed a new general HPV DNA real-time PCR on the Mx4000 (Stratagene) and LightCycler (Roche Diagnostics) systems usable for both cervical scrape specimens and urine samples. A linear range was obtained from 5 DNA copies to 8 log₁₀ DNA copies/ml, and intra- and interassay variations were between 1.8 and 4%. Cervical carcinoma and HPV DNA screening was performed in 333 individual women referred for gynecological examination at the university hospitals of Angers and Brest and enrolled in the PapU study. Among cervical specimens (*n* = 333), 45% were positive for HPV DNA, with a mean viral load at 5.00 log/ml (± 1.73). Among urine samples (*n* = 177), 37% were positive with a significant 50-fold-lower mean viral load (3.77 ± 1.32 log/ml; *P* < 0.0001). Kappa agreement for HPV DNA between cervical and urine specimens was excellent (93%). Thus, we developed a highly sensitive and quantitative general HPV DNA real-time PCR method that allows mass screening of patients with HPV infection. The ongoing longitudinal and prospective multicenter PapU study should give us the opportunity to validate this method adapted to HPV DNA screening in urine samples in a larger population.

Human papillomaviruses (HPVs) are epitheliotropic viruses associated with benign and malignant lesions of cutaneous and mucosal epithelia. More than 100 different types of HPV have been identified to date, of which 40 have been reported in anogenital infections. In a recent multicenter analysis involving 1,918 women in 11 case-control studies (14), 15 HPV genotypes (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) were classified as high risk (HR-HPV) and associated with precancerous lesions of the cervix, 3 were classified as probable HR-HPV (types 26, 53, and 66), and 12 were classified as low risk (LR), i.e., not associated with the development of cervical carcinoma (types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108). Because of the strong association between HPV infection and cervical cancer, detection of HPV DNA in cervical samples may be an option for identifying women at risk of developing cancer (13). However, cervical sampling is uncomfortable, time-consuming, and requires a degree of skill. Self-collected cervical sampling was not found to be as efficient as sampling done by a physician (19). Therefore, about 40% of the women in France presenting a cervical carcinoma have never been screened. Moreover, it would be easier to use urine specimens as is done with molecular detec-

tion of *Chlamydia trachomatis* (7, 21). This would simplify mass screening and survey of HR-HPV female carriers.

Efficient HPV culturing remains elusive, and the clinical performance of serological assays is still poor. Thus, diagnosis of HPV infection is based almost entirely on molecular tools, including liquid hybridization (e.g., hybrid capture), Southern and dot blot hybridization with HPV type-specific probes, type-specific PCR, and general-primer PCR. Several general PCR primers have been developed to detect a broad spectrum of HPV genotypes. The majority of large studies to date have been performed with the MY 09/11, the GP5+/6+, and the SPF10 general primer sets allowing the amplification of the L1 HPV region. Various methods have been described for detection and identification of HPV genotypes after amplification with general PCR primers, such as the Amplicor HPV assay combined with linear array (Roche Diagnostics) or the SPF10-Line probe assay (LiPA; Innogenetics) showing similar results (10, 15, 18, 19, 23, 24).

In this study, we propose a new, highly sensitive, real-time general PCR that will allow quantification and typing of more than 50 HPV genotypes. This method can also be used for urine samples, permitting mass screening of HPV genital infections.

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MATERIALS AND METHODS

Patients and specimen collection. Cervical scrape samples were collected from women consulting a gynecologist at the following units: the Department of Obstetrics and Gynecology of the Angers University Medical School Hospital,

TABLE 1. Sequences of HPV general primers derived from SPF10 primers and of GAPDH DNA cell primers and TaqMan probe^a

Primer	Sequence ^b
Forward HPV primers	
HP 1E	5'-AACGTGCICAGGGICACAATAATGG-3'
HP 1F	5'-ATAAGGCICAGGGICATAACAATGG-3'
HP 1G	5'-AACGTGCICAGGGICATAATAATGG-3'
HP 1H	5'-AACGTGCICAAGGICATAATAATGG-3'
Reverse HPV primers	
HP 2Cbiot	5'-biotCGGGTAGTATCIAACAACAGTAACAAA-3'
HP 2Dbiot	5'-biotCGAGTGGTATCTACCACAGTAACAAA-3'
HP 2Ebiot	5'-biotCGGGTAGTATCIACTACAGTAACAAA-3'
GAPDH	
Forward primer	5'-GGACTGAGGCTCCACCTTT-3'
Reverse primer	5'-GCATGGACTGTGGTCTGCAA-3'
HT probe	5'-FAM-CATCCAAGACTGGCTCCTCCTGC-TAMRA-3'

^a SPF10 primers are from reference 9.

^b biot, 5'-end-labeled biotinylated primers; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

the Department of Obstetrics and Gynecology of the Brest University Medical School Hospital, the Angers Antivenereal Dispensary, and the Angers Women and Children Protection Unit. The samples were prospectively assessed for HPV screening. Patients were proposed participation (with informed consent) in the PapU study, a prospective longitudinal multicenter study for HPV DNA detection in urine and cervical samples, that started in 2004 and is currently under way (up to 2007). HPV-positive patients were invited for a follow-up visit after 6 to 12 months. Both cytobrush of cervical scrapes in 2SP (2 M sucrose phosphate) medium (2 ml) and, when included in our study, first-stream urine (5 to 10 ml) specimens were sampled for each patient and stored at -80°C until analysis.

DNA isolation. DNA was extracted from 200 μl of cervical samples using a QIAamp DNA mini kit (QIAGEN, Courtaboeuf, France), as recommended by the manufacturer. Briefly, sample lysis was obtained by proteinase K digestion at 56°C for 10 min, and the lysate was then loaded on the QIAamp column. After two washes, DNA was eluted with 200 μl of elution buffer provided in the kit. For DNA extraction from urine, a QIAamp viral RNA mini kit from QIAGEN was used. As recommended by the manufacturer, 1 ml of urine was centrifuged for 1 h at $23,000 \times g$, and the pellet was resuspended with 200 μl of sterile phosphate buffer. After lysis, the suspension was loaded onto the QIAamp column; DNA was then washed twice and eluted with 50 μl of elution buffer. Extracted DNA was kept at -80°C before PCR analysis.

Plasmid and cell controls. HPV type 6 plasmid (pHP6) was previously constructed in the Angers virology laboratory using the pMos Blue vector (Amersham, France). A 73-base amplified DNA in the L1 region was introduced in the unique EcoRV restriction site of pMos Blue. The insertion sequence was controlled by sequencing pHP6 using the Ceq2000 capillary system (Beckman), and BLAST analysis was performed on the NCBI website. Plasmid concentration was measured by DNA spectrophotometric analysis at 260 nm (160 ng/ μl or 5×10^{10} DNA copies/ μl).

The SiHa cell line, kindly provided to the Brest virology laboratory by Marianne Coste (Virology Laboratory, Nantes, France), is considered to contain one to two copies of HPV16 per cell (average of 1.5 copies according to our calculation). These cells were cultured in minimal essential medium and 10% fetal calf serum, trypsinized at confluence, and pelleted; cells were then resuspended in sterile phosphate buffer to get 10^7 HPV DNA copies per ml. Five 10-fold serial dilutions in phosphate buffer from this suspension were done. DNA was extracted from these pure and diluted cell suspensions with the QIAamp DNA mini kit as for cervical samples.

We used pHP6 and SiHa cells to assess the sensitivity and the reproducibility of our method.

Real-time PCR. HPV DNA amplification and quantification were carried out in the Mx4000 (Stratagene Europe, Amsterdam, The Netherlands) and in the LightCycler (Roche Diagnostics, Meylan, France), used, respectively, in the Angers and Brest virology laboratories. An SYBR green protocol was assessed on these instruments with modified SPF10 general primers (Table 1) allowing detection of about 40 HPV genotypes (9). Modification with 4 more bases at the 5' end of the original SPF primers adjusted to several HPV genotypes allowed hybridization at 60°C and a two-amplification step protocol at 60 and 95°C for

real-time PCR. The addition of a biotin in the 5' end of reverse primers allowed direct typing of HPV-positive samples using the LiPA. Forward and reverse modified SPF primers were pooled at 2.5 pmol/ μl . Five microliters of extracted DNA from samples was added to SYBR green master mix (Stratagene for Mx4000 or Roche Diagnostics for LightCycler) with forward and reverse modified SPF primers at 100 nM in a final volume of 25 μl for Mx4000 or 20 μl for the LightCycler. Thermal cycling was one step at 95°C for 10 min, followed by 45 cycles at 95°C for 30 s and 60°C for 1 min using the Mx4000 or 95°C for 15 s and 60°C for 30 s using the LightCycler. A melting curve was added between 55 and 95°C . The expected melting temperature for HPV L1 SPF amplicons was around 72°C ($\pm 1^{\circ}\text{C}$) using Mx4000 and 77°C ($\pm 1^{\circ}\text{C}$) using LightCycler.

Cell glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA from each sample was tested in parallel, as internal control and for cell number evaluation, in the same thermal run with the TaqMan universal master mix (Applied Biosystems), forward and reverse GAPDH primers at 300 nM, and a 6-carboxyfluorescein-6-carboxytetramethylrhodamine GAPDH probe at 250 nM (Table 1). Results were expressed in threshold cycle number (C_T), the number of cycles at which the fluorescent signals entering the log-linear phase were consistently obtained. The number of cells in each sample was estimated by the following calculation obtained after several cell counts and GAPDH C_T measurements: $n(\text{cells}) = 10^{\text{EXP}}[(-0.2861 \times \text{GAPDH } C_T + 12,602) - 1]$; the correlation coefficient, r , has a value of 0.96.

Series of 10-fold diluted SiHa cells and diluted pHP6, corresponding to, respectively, 5, 50, 500, 5,000, and 50,000 DNA copies of HPV16 and 25, 250, 2,500, 25,000, and 250,000 DNA copies of HPV6 were run in parallel to plot the standard curves for HPV DNA quantification in samples. Triplicate dilution series were included as standards in each run. The HPV copy number in samples was evaluated using regression analysis. Corresponding C_T values were reported to the standard curve allowing sample results expressed as negative (no C_T) or positive, and in the latter, quantitatively in \log_{10} DNA copies/ml of cervical medium or urine. All assays were performed in duplicate on the Mx4000 and in a single test on the LightCycler.

Optimization of our protocols on the Mx4000 and LightCycler was obtained by evaluation of primer concentrations in a range of 100 to 300 nM, MgCl_2 concentration for LightCycler master mix from 3 to 5 mM, and thermal hybridization temperature between 52 and 62°C on Mx4000 in 10-fold diluted pHP6 and SiHa cells as DNA standards in triplicate.

Genotyping using reverse hybridization by the INNO-LiPA HPV genotyping system. Twenty microliters of the biotinylated real-time PCR product from positive samples was added to the denaturation solution and incubated with one strip in hybridization solution from the INNO-LiPA kit (Innogenetics, Ghent, Belgium). Each membrane strip contained 25 oligonucleotides specific for 25 different HPV types (namely, types 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68/73, 70, and 74). The strips were washed twice under stringent conditions and then incubated with an alkaline phosphatase-labeled streptavidin conjugate for color development by the addition of 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium in substrate buffer. After a final washing and drying, the strips were visually interpreted using a grid as previously described (10).

Statistical analysis. Qualitative parameters were analyzed by using the Fisher exact test, and quantitative parameters were analyzed by using the Mann-Whitney nonparametric test and Pearson's correlation factor. The sensitivity of HPV detection in urine compared to cervical samples was expressed in percentages, with 95% exact confidence intervals. Cohen's kappa constant was calculated to evaluate the measure of kappa agreement (κ value) for HPV detection between urine and cervix. A P value higher than 0.05 and a κ value higher than 0.60 were considered significant. All statistical analysis was performed using the software SPSS, version 10.1, for Windows (SPSS Inc., Chicago, IL).

RESULTS

Optimization of our general HPV protocol. The best primer concentrations were defined at 100 nM with 98.8% efficacy. Increasing the reverse-pooled primer concentration did not modify C_T results of diluted pHP6 and SiHa extracted DNA, but using forward-pooled primer concentrations higher than 200 nM showed nonspecific positive C_T results. Biotinylation of reverse primers did not affect PCR efficacy compared to non-biotinylated primers (respectively, 98.8 versus 99.1% efficacy; $P > 0.05$). Hybridization temperature assays between 52 and

TABLE 2. Linearity and reproducibility of general HPV DNA real-time PCR on the Mx4000 and LightCycler

Parameter	Value(s) with HPV DNA protocol	
	Mx4000	LightCycler
Threshold (HPV DNA copies/assay)		
50%	15	25
95%	150	200
Linearity (log ₁₀ DNA copies/ml)	1.17–8.39	1.39–7.69
Reproducibility at 500 copies (mean C _T , SD, CV [%]) ^a		
Intra-assay	31.62, 0.58, 1.8	31.65, 0.69, 2.2
Interassay	32.59, 0.65, 4.0	33.19, 0.70, 3.2

^a SD, standard deviation; CV, coefficient of variation.

62°C gave specific results over 58°C. Below this level, nonspecific positive results were observed. A temperature of 60°C was found to be most appropriate for the thermal cycling program. The best MgCl₂ concentration was defined at 4 mM for the LightCycler protocol. A 5 mM concentration gave similar C_T results, but a 3 mM concentration gave higher C_T levels but with lower sensitivity (about fivefold).

Development of real-time PCR to amplify and quantify HPV DNA. Mx4000 and LightCycler protocols gave very similar results (Table 2). Threshold levels of 50 and 95% of positive samples were evaluated using pHP6 and SiHA cells diluted 10-fold and 4-fold (Table 2). The lowest detection threshold was defined at about 5 HPV DNA copies. Linearity assays were obtained up to 8 log₁₀ DNA copies/ml. The reproducibility of inter- and intra-assay results upon 10 runs was below 5%. The slope was excellent (−3.22), showing high efficiency (98%). Results for pHP6 and SiHa cells containing, respectively, HPV6 and -16 types were similar (C_T at 31.65 versus 31.37 for 500 HPV DNA copies and C_T at 28.63 versus 28.28 for 5,000 HPV DNA copies).

Evaluation of HPV DNA quantitative real-time PCR on clinical samples. Cervical scrape samples were obtained from 333 women followed in the Angers and Brest hospitals. Among them, 177 participated in the PapU study. A panel of 10 positive samples was evaluated by both the Mx4000 and LightCycler protocols. Median values were comparable (4.83 log DNA copies/ml for Mx4000 versus 5.04 log/ml for LightCycler, with a mean difference of 0.21 log and a Pearson’s correlation of 0.79), confirming our plasmid results. All cervical samples were positive for GAPDH DNA, showing the presence of cells in all samples (C_T = 25.60 ± 5.66, corresponding to about 620,000 cells with a range from 10³ to 7 × 10⁶ cells). Most of the urine samples presented cells (150 out of 177 had a positive GAPDH C_T with an average C_T at 29.71, corresponding to about 16,000 cells, with a range from 0 to 3 × 10⁵ cells).

HPV-positive samples were found in 150 cervical specimens out of 333 (45%) and 66 urine samples out of 177 (37%). Good agreement was obtained for HPV detection in cervical and urine samples, with a kappa test at 93% (Table 3). However, HPV viral load was 50-fold higher in cervical samples than in urine (respectively, 5.00 ± 1.73 versus 3.77 ± 1.32 log/ml; *P* < 0.0001). Levels of viral load in cervical specimens were correlated to levels in urine (*r* = 0.81; *P* = 0.009). Discordant results between positive cervical and negative urine samples more

TABLE 3. Overview of the 177 patients tested for HPV DNA in urine and cervical paired samples^a

Test result	No. of samples ^a		
	Positive CS	Negative CS	Total
Positive urine	62	4	66
Negative urine	6	105	111
Total	68	109	177

^a Sensitivity with urine, 91.2%; specificity with cervical samples (CS), 96.3%; agreement, 94.4%; kappa test, 93.2%.

often showed low viral loads (below 3 log/ml). No correlation was found between viral load in both samples and levels of cells (41% of cervical specimens and 62% of urine samples were not correlated to cell levels estimated by GAPDH C_T levels; *r* = 0.64 and 0.54, respectively; *P* > 0.05).

The viral load observed in high-grade squamous intraepithelial lesions (HGSIL) was higher than that of normal cytology (respectively, 5.52 ± 1.95 versus 4.26 ± 1.42 log/ml; *P* = 0.008). HR-HPV type 16 was found most frequently (34.9%), followed by type 66 (14.9%), type 31 (10.8%), type 33 (9.5%), type 56 (8.6%), and type 18 (6.8%). Mixed types accounted for 31.7% of the patients with always one or more HR-HPV types, whereas LR-HPV types alone were found in 8.7% of cases. Higher viral load was observed with HR-HPV types than with LR-HPV types (5.52 ± 1.95 versus 4.26 ± 1.42 log/ml, *P* = 0.01). Among HR-HPV types, the highest viral loads were observed with types 16, 18, 31, and 33 (5.99 to 6.49 log/ml) compared to types 56 and 66 (5.17 to 5.29 log/ml; *P* = 0.02).

DISCUSSION

We have reported herein a highly sensitive (about 5 copies), linear (up to about 100 million copies), and quantitative real-time general PCR for HPV DNA detection. This method, on which relies the diagnosis of HPV infection, was adapted to two commonly used real-time PCR systems (LightCycler from Roche Diagnostic and Mx4000 from Stratagene) with comparable results. Previous studies have suggested that methods with higher sensitivity could make HPV detection in urine possible (2, 3, 6, 20). The urine specimen approach should facilitate HPV detection in women who do not have access to a gynecologist and should be more attractive to patients whatever their social origin. HPV-positive patients (about 10% expected) would then be referred to a gynecologist for clinical and cytohistological diagnosis of the cervix.

Therefore, we have adapted the previously described SPF10 PCR method (9) to real-time PCR, which shows higher sensitivity in the detection of more HPV infections from almost 57 different genotypes, compared to two other general PCR-based methods using the GP5+/6+ and MY09/11 primer sets. These primers (SPF, GP, and MY) allowed amplification of different overlapping fragments in the HPV L1 region between nucleotides 6500 and 7000 from HPV with, respectively, 65, 150, and 450 bp. The smallest fragment obtained with the SPF10 primers was the most appropriate for real-time PCR thermal conditions, since only small PCR fragments are required. Also, a type-specific assay is possible using the SPF10

amplicons with biotinylated reverse primers on the LiPA system developed by Innogenetics (10). We showed in this study that the use of biotinylated primers in real-time PCR did not affect amplification and SYBR green detection. We also optimized the SPF10 primers to allow a 60°C hybridization temperature as the optimal thermal conditions for real-time PCR.

A highly sensitive method may be required in order to realize the highest negative predictive value for detecting HPV-associated cervical carcinoma (i.e., no detected HPV is related to no risk of developing a carcinoma). Amplification detection assays, such as PCR-based methods, are more sensitive than liquid hybridization tests such as the Digene Hybrid Capture II (HC II) assay, as demonstrated previously for several viral molecular markers, such as cytomegalovirus DNA, hepatitis B DNA, and more recently HPV DNA (15). Indeed, Perrons et al. (15) showed that the HC II assay could detect only 56% of positive HR-HPV DNA using the SPF10 PCR method tested in parallel, whereas the latter could detect all HC II-positive HR-HPV. They also demonstrated that lower sensitivities of the HC II assay were obtained for Pap smears classified as normal or as atypical squamous cells of undetermined significance (respectively, 28 and 45% compared to 55 and 69% using the SPF10 PCR method), related perhaps to lower viral load in these groups of patients. The HC II assay is reported to have an analytical sensitivity at 1 pg of HPV DNA, corresponding to about 5,000 DNA copies, and to be less sensitive than PCR methods which allowed cutoff levels of about 5 to 500 copies (12). However, PCR methods may be impeded by inhibitors, as shown in cervical lavage samples (11), urethral swab specimens (22), or urine samples (3). It is important to evaluate the efficacy of the PCR using internal controls and to use the best DNA extraction procedure. Therefore, we used the GAPDH DNA cell control to assess the presence of cells in samples and elimination of inhibitors, which have been found in 7 to 19% of endocervical samples and about 50% of urethral and urine samples. GAPDH DNA has been found to be a stable marker for cell counting estimation (8). Other cell markers, such as β -globin, were shown to be insensitive to some inhibitors in 8% of cervical lysates (11). Appropriate DNA extraction procedures have been shown to eliminate these inhibitors, as demonstrated by Brinkman et al. for HPV extraction in urine samples (3). Those authors found that urine could inhibit HPV DNA amplification 10⁶-fold more than water by using a type 16 HPV diluted plasmid. They showed that by using the silica-based (1) viral DNA/RNA extraction method from QIAGEN, as we used in this study, they were able to reduce the inhibitor effect more than 10⁵-fold.

A reliable HPV DNA quantification method is required for viral load measures. High viral load seems to be indicative of viral persistence and disease development, whereas low viral load is more often associated with viral clearance (5, 21). Although the HC II assay has not been validated as a quantitative test, it was shown in these studies that a high ratio of HPV to relative light units was related to the risk of developing HGSIL. However, more-precise methods have to be evaluated for this purpose. The recent availability of real-time PCR methods allows such an approach (17). However, clinical viral load thresholds have to be defined. Using a specific HPV type 16 quantitative method, Pretet et al. (17) showed that a viral load higher than 10⁷ copies per ml may contribute to lesion

evolution towards cervical intraepithelial neoplasia 2 to 3, corresponding to more than 100 pg/ml using the HC II test. We also observed that a higher viral load in patients (over 10⁵ copies per ml) was related to HGSIL. Variation in the clinical threshold emphasizes the need of standardization of the methods with a control panel (18). The HC II assay is the only U.S. Food and Drug Administration-approved method, and the lack of a reference method makes the constitution of a control panel difficult. We also showed in this study that viral load was not correlated to cell levels. Therefore, we do not agree with Pretet and collaborators, who chose to normalize HPV copy numbers according to sample cellularity assessed by albumin PCR (17). Those authors found good correlations between their method and the HC II assay, whether HPV DNA was expressed in copies per ml or per 10³ cells. However, we did not find a correlation when HPV DNA was expressed in copies per ml or per 10² cells. Indeed, we observed samples, mostly urine but also cervical specimens, with high viral load and few cells. Normalization of the HPV value might contribute to under- or overestimation of the viral load.

Finally, it is important to express HPV results in terms of specific genotype. Indeed, the recent study from Perrons et al. (15) showed that among followed HPV-positive patients assessed by the SPF10-LiPA method (60% of the included patients), 28% were positive with the same genotype at 6 months and 7% were positive with a different genotype; the first situation would be considered a persistent infection whereas the second would be seen as a "resolved-acquired" infection. Both situations were considered persistent infections by using the HC II assay. These investigators also observed, as did others (4, 16), cross-reactivity between HR- and LR-HPV; 13 out of 20 LR-HPVs assessed by the SPF10-LiPA system were found to be HR-HPV using the HC II assay. Cross-hybridization has been found with at least 22 different LR-HPV types with the current HR-HPV HC II probes (16).

In conclusion, we developed a highly sensitive and quantitative general HPV DNA real-time PCR method that allows mass screening of patients for HPV infection. This method allows specific genotyping using the LiPA system. Our method also seems to be sensitive enough to allow HPV detection in urine samples with high performance, owing to improvement of the whole PCR process, as suggested by the recent study of Daponte et al. (6). The ongoing longitudinal and prospective multicenter PapU study should give us the opportunity to validate this approach by using this method in a larger population and in the new age of vaccination against HPV infections.

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