High Level of Correlation of Human Papillomavirus-16 DNA Viral Load Estimates Generated by Three Real-time PCR Assays Applied on Genital Specimens

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Abstract

Human papillomavirus-16 (HPV-16) viral load could be a biomarker predictive of the presence of high-grade cervical lesions. Recently, several real-time PCR assays have been developed to accurately measure HPV-16 viral load. However, results from various reports using these assays cannot be compared because interassay test correlation has not been documented. The variability of HPV-16 DNA quantitation was assessed by comparing three real-time PCR assays (HPV-16 L1, HPV-16 E6, and HPV-16 E6 PG) applied on 144 genital samples (125 cervicovaginal lavages and 19 specimens collected using vaginal tampons) obtained from 84 women (66 HIV seropositive and 18 HIV seronegative). Correlation was greater between the HPV-16 E6 assays [correlation coefficient (ρ) = 0.92] than between each E6 assay and HPV-16 L1 assay (ρ = 0.83 and 0.84, respectively). The median HPV-16 copies measured by HPV-16 E6 PG (14,609 HPV-16 copies/2 μL sample) and HPV-16 E6 (18,846 HPV-16 copies/2 μL) were similar (P = 0.27) but were both greater than the median HPV-16 copies measured with the L1 assay (4,124 HPV-16 copies/2 μL; P < 0.001). Correlations between HPV-16 E6 assays were similar for samples containing non-European (ρ = 0.93) or European (ρ = 0.95) variants. However, the correlation between HPV-16 L1 and HPV-16 E6 PG or HPV-16 E6 was lower for specimens containing non-European variants (ρ = 0.80 and 0.76, respectively) compared with specimens containing European variants (ρ > 0.85). HPV-16 DNA quantity estimated with the three assays was comparable although lower with the HPV-16 L1 assay. The level of correlation depended on viral polymorphism, viral load, and cervical disease status. (Cancer Epidemiol Biomarkers Prev 2005;14(9):2200–7)

Introduction

Anogenital human papillomaviruses (HPV) cause cervical intraepithelial neoplasia (CIN) and cancer of the uterine cervix (1-3). HPV-16 is the most frequent genotype detected in sexually active women without lesion as well as in women with invasive cervical cancer and high-grade CIN in North America (4-6). Because of the high cumulative prevalence of HPV-16 infection in sexually active women and of the low prevalence of CIN (7), an important proportion of HPV-16-infected women is free of cervical disease. To better identify women infected by HPV who have underlying cervical disease, biomarkers related to HPV infection have been evaluated in cross-sectional and cohort studies. Several studies have suggested that increased high-risk HPV DNA load could be such a marker for the presence of CIN in HIV-seronegative and HIV-seropositive women (8-16). Moreover, high HPV-16 load in women with normal cytopathology smears could be a determinant for neoplastic progression (8, 10, 17).

However, viral load measures are modulated by several factors that impede the direct comparison of studies. For example, the presence of surrounding low-grade lesions strongly affects cervical HPV viral load measures in women with high-grade CIN (18). Several studies used semiquantitative PCR assays that measured the amount of HPV DNA over a limited range of concentrations. HPV viral loads were also measured in some studies with the Hybrid Capture 2 assay, a semiquantitative signal amplification test detecting 13 high-risk and 5 low-risk HPV types (19-26). However, the latter assay does not measure HPV DNA from individual types and has a sensitivity end point of 5,000 HPV DNA copies per test (26, 27). Furthermore, it may be important to control for the cell content of samples to express HPV viral load as the number of HPV copies per microgram of cellular DNA (9, 12, 28). In contrast to these findings, two studies reported that HPV DNA quantities measured with Hybrid Capture 2 were independent of sample cellularity (29, 30). Until these issues are resolved, HPV viral load assays should measure and report viral and cellular content quantitatively in samples.

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Real-time PCR assays can accurately and rapidly quantitate DNA molecules over a wide dynamic range. They attain levels of sensitivity near one copy per test and have been shown to be very specific (9, 31-35). Real-time HPV-16 PCR assays normalizing for total cell number have been developed for the investigation of evolution of HPV viral load in the natural history of CIN (8-10, 13, 15, 28, 33, 36-38). The intralaboratory and interlaboratory reproducibility of these assays is very good to excellent (31-33). Nevertheless, the comparability of HPV DNA quantitation results determined by different real-time PCR assays is unclear. This information is essential for the interpretation of differences between studies.

Given the potential of real-time PCR assays as exposure assessment tools for the investigation of the role of HPV DNA viral load in the natural history of HPV-induced lesion, the present investigation was conducted to assess correlation between measures of HPV-16 DNA copy numbers and HPV-16 viral loads obtained by three different real-time PCR assays applied on 144 HPV-16-positive samples collected in a prospective cohort study. Factors that could affect levels of correlation between assays are described.

Materials and Methods

Study Population and Study Design. One hundred forty-four samples were derived from a bank of 403 HPV-16-positive genital specimens that had been collected between May 1993 and March 2002 from 132 women with persistent or transient HPV-16 infection participating in the Canadian Women's HIV Study (13, 39). The Canadian Women's HIV Study is a cross-sectional and cohort study that investigates determinants of HPV infection and persistence in 1,055 women infected or at risk for HIV (39-41). Women were enrolled from sexually transmitted diseases, primary care clinics, and outpatient HIV care clinics across Canada. They were eligible if they provided written consent and either were seropositive for HIV or were seronegative for HIV but at risk for sexually transmitted infections (39, 41).

At recruitment and follow-up visits scheduled at 6-month intervals, a vaginal tampon specimen was obtained as described previously (40) followed by a cervical Papanicolaou smear obtained with a cytobrush and Ayres spatula. Then, a cervicovaginal lavage was done with 10 mL sterile PBS (40). Cell suspensions were lysed with 0.8% Tween and digested with proteinase K. An aliquot of 5 μL from each processed sample was amplified for β-globin DNA with PC04-GH20 (40). β-Globin-positive samples were tested for HPV DNA detection and typing using the MY09-MY11-HMB01 consensus L1 PCR and type-specific probes (40, 42). Cytology smears were interpreted in one central pathology laboratory using the 1991 Bethesda classification (43). Colposcopy and biopsy results were made available to the study investigators. Ethics committees of each participating institution approved the study protocol.

Panel of Samples Analyzed by Real-time HPV-16 PCR Assays. One hundred forty-four HPV-16-positive samples (125 cervicovaginal lavages and 19 specimens collected using vaginal tampon) were selected for the current evaluation as described above. These samples had been screened in previous studies for the presence of inhibitors by real-time PCR using internal controls for HPV-16 and β-globin (28, 31, 32). Briefly, 1,000 copies of HPV-16 L1 internal control, 1,000 copies of HPV-16 E6 internal control, and 1,000 copies of β-globin internal control were amplified in separate capillaries with 2 μL specimen lysate and tested in a Light Cycler PCR and detection system (Roche Molecular Systems, Branchburg, NJ; refs. 28, 31, 32). The presence of PCR inhibitors was suspected when 1,000 copies of at least one of the internal controls generated a signal corresponding to <700 copies as explained in detail previously (31). Nineteen samples containing inhibitors [inhibition of all internal controls ($n = 4$) and inhibition of HPV-16 internal controls only ($n = 15$)] were tested after a 1:10 to 1:1,000 dilution of lysate ($n = 12$) or after DNA purification with Master Pure ($n = 7$; refs. 31, 44). No sample showed selective inhibition against only one of the two HPV-16 internal controls. Two microliters of each sample were tested blindly in duplicate with real-time PCR assays HPV-16 L1 and HPV-16 E6 in laboratory A and HPV-16 E6 PG in laboratory B (see below).

HPV-16 L1 Real-time PCR. The 20-μL reaction mixture contained 1× DNA Master Hybridization Probe Mix (Roche Molecular Biochemicals, Laval, PQ) comprising the FastStart Taq DNA polymerase, 4.5 mmol/L $MgCl_2$, 0.3 μmol/L of each HPV-16 L1 primers U6564 and L7012, and 50 nmol/L probe U6862 labeled with FAM (9, 31, 32). After Taq polymerase activation for 7 minutes at 95°C, amplification was done for a total of 50 cycles in the Light Cycler system at 95°C for 15 seconds, 60°C for 5 seconds, and 65°C for 60 seconds (31). The first cycle in which the logarithmic linear phase of fluorescence could be distinguished from background was designated the threshold cycle (C_t). Quantitation was accomplished via extrapolation from an external standard curve of serial 10-fold dilutions of a HPV-16 DNA plasmid (kindly provided by Prof. zur Hausen, Germany) in 10 mmol/L Tris-HCl (pH 8.2). A linear regression was fit to each curve and the resulting regression equation was used to calculate unknown starting target concentration based on the measured C_t values.

HPV-16 E6 PCR. The 20-μL reaction mixture contained 1× DNA Master Hybridization Probe Mix as above, except for the use of 4 mmol/L $MgCl_2$, 0.3 μmol/L of each HPV-16 primers 16-E6-F and 16-E6-R, and 50 nmol/L probe16-E6-PRO (16, 28). After Taq polymerase activation for 7 minutes at 95°C, clinical samples and aliquots from the titration curve used for HPV-16 L1 quantitation were amplified in a Light Cycler system with the same cycling variables as for HPV-16 L1.

HPV-16 E6 PG PCR. The 20-μL reaction mixture contained 10 mmol/L Tris-HCl (pH 8.0), 50 mmol/L KCl, 200 μmol/L of each dATP, dGTP, and dCTP, 400 μmol/L dUTP, 0.1 μmol/L TaqMan probe HPV-16-TM, 0.2 μmol/L of each primer HPV-16-U and HPV-16-L in E6, 4 mmol/L $MgCl_2$, and 5 units AmpliTaq Gold DNA polymerase (29, 33). Following a brief centrifugation, the reaction plate was placed in an ABI 5700 Sequence Detection System and amplified at 50°C for 2 minutes and 95°C for 12 minutes followed by 50 cycles at 95°C for 15 seconds and 55°C for 30 seconds. The external standard curve was made of serial dilution of HPV-16 DNA extracted from SiHa cell line.

β-Globin PCR. β-globin gene was amplified with 50 nmol/L β-globin probe U62049, 0.3 μmol/L of primers U61992 and L62240, 4.5 mmol/L $MgCl_2$, and 1× DNA Master Hybridization Probe Mix. After Taq polymerase activation for 10 minutes at 95°C, samples were amplified for 50 cycles at 95°C for 15 seconds and 60°C for 60 seconds. Titration curves of human DNA were obtained by serial dilutions of a stock of human placental DNA D4642 (Sigma, St. Louis, MO) in 10 mmol/L Tris-HCl (pH 8.2).

ERV-3 PCR. Quantitation of cellular DNA was conducted by ERV-3 real-time PCR assay as described previously in an ABI 5700 Sequence Detection System (45) with a master mix as described for HPV-16 E6 PG PCR, except for the use of 0.25 μmol/L TaqMan probe PHP-P505, 0.2 μmol/L of each PHP10-F and PHP10-R primers, and 5 mmol/L $MgCl_2$. Samples were amplified at 50°C for 2 minutes and 95°C for 10 minutes followed by 45 cycles at 95°C for 60 seconds and 58°C for 60 seconds. Cellular controls were generated from serial dilution of human DNA from the K562 cell line in a background of 5.0 μg/mL yeast tRNA in TE.

Molecular Variant Analysis by PCR Sequencing. HPV-16 isolates were further characterized by PCR sequencing of a 364-bp segment within the LCR (46) and the complete E6 gene (47). HPV-16 amplicons were generated with 2.5 units Expand High Fidelity PCR enzyme (Boehringer Mannheim, Laval, Quebec, Canada) and purified with the QIAquick gel extraction kit protocol (Qiagen, Inc., Mississauga, Ontario, Canada). Direct double-stranded PCR-sequencing was done with the fluorescent cycle sequencing method (BigDye Terminator Ready Reaction kit, Perkin-Elmer, Montreal, PQ) on an ABI Prism 3100 Genetic Analyzer System. HPV-16 isolates were classified into two broad categories (prototype and nonprototype strains) and into one of the five lineages as described by Yamada et al. (48).

Analysis of Results and Statistical Methods. Quantities of HPV-16 L1 and E6 DNA were expressed as the number of HPV-16 DNA copies per 2 μL sample. In a subset of 40 samples for which cellular quantity was measured, HPV-16 L1 and E6 viral loads were expressed as the number of HPV-16 copies per microgram of human DNA. Scatter diagrams plotting mean HPV-16 DNA quantity of duplicates for each assay were generated. Relative correlation between HPV-16 DNA copy numbers or viral loads estimated with each real-time PCR assay was assessed by the Spearman's rank correlation coefficient (ρ). Pearson's correlation coefficient (r) was also calculated to measure the linearity of the relationship between two sets of measures of HPV-16 DNA. The Kolmogorov-Smirnov test was applied on the measures of HPV-16 DNA copies obtained with the L1 assay to assess if they were distributed normally. HPV-16 DNA copies measured with the three real-time PCR tests were compared with the Sign test. The magnitude of the association between ethnicity and HPV-16 LCR polymorphism was assessed by logistic regression controlling for age and a factored variable combining CD4 cell count and HIV status (HIV-negative and HIV-positive with CD4 >500, 200-500, and <200 cells/μL). HPV-16 viral loads and ratios of HPV-16 quantities measured with HPV-16 E6 and HPV-16 E6 PG assays from isolates of different HPV-16 E6 lineages were compared with the Mann-Whitney U test. All statistical tests were two sided with statistical significance set at $P < 0.05$. Cross-tabulations of data, Spearman's and Pearson's coefficients, and statistical significance were determined with Statistica version 6 software (StatSoft, Tulsa, OK).

Results

HPV-16 DNA was measured in 144 processed genital samples collected from 84 women (mean, 1.8 ± 1.2; median, 1.0; range, 1-6 specimens per woman). A single sample was obtained from 54 participants and two to six samples were obtained at 6-month intervals from 30 women. The 66 HIV-seropositive and 18 HIV-seronegative women provided 116 and 28 genital specimens, respectively. The demographic and virological characteristics of our patient population are described in Table 1. Samples testing negative for HPV-16 in at least one real-time PCR assay were often positive in the other assays but had a low HPV-16 copy number (Table 2). Four samples tested negative for HPV-16 DNA in all real-time PCR assays (Table 2).

The duplicates showed good intralaboratory (intrarun) variability with median coefficients of variation of 14.5%, 6.7%, and 4.4% for HPV-16 L1, HPV-16 E6 PG, and HPV-16 E6 real-time PCR assays. Very high levels of correlation were obtained between duplicates for HPV-16 L1, E6, and E6 PG assays with ρ coefficients of 0.97, 0.99, and 0.98, respectively. We then correlated HPV-16 DNA copy numbers in 2 μL of processed samples measured with each of three real-time PCR assays. Unfortunately, 2 of the 144 samples did not contain enough material for the HPV-16 E6 PG assay and were excluded from the analysis, leaving 142 samples for the comparison. The samples analyzed showed a wide distribution of number of HPV-16 DNA copies (Fig. 1). There was no difference in HPV-16 viral load measurements in specimens that needed to be diluted or purified compared with those tested after cell lysis only ($P = 0.89$; data not shown). The $\log_{10}$ HPV-16 DNA copy values were plotted for each assay against the others (Fig. 2). Linear correlation between each pair of tests suggested good to excellent agreement with Pearson's correlation coefficients from 0.78 to 0.89. HPV-16 copies measured with HPV-16 E6 strongly correlated with the HPV-16 E6 PG assay [$\rho = 0.92$; 95% confidence interval (95% CI), 0.89-0.95]. As shown in Fig. 2, correlation between each HPV-16 E6 assay and the HPV-16 L1 assay [$\rho = 0.83$ (95% CI, 0.78-0.88) and 0.84 (95% CI, 0.79-0.89), respectively] was significantly weaker than correlation between E6 assays ($P = 0.001$).

Slopes obtained from standard curves of prototypic HPV-16 DNA tested thrice were similar for each assay *in vitro* (−3.38 for HPV-16 E6, −3.32 for HPV-16 E6 PG, and −3.22 for HPV-16 L1). The median HPV-16 copies/2 μL sample measured by HPV-16 E6 PG, HPV-16 E6, and HPV-16 L1 assays reached 14,609 HPV-16 DNA copies (mean, 7,668,954 ± 63,847,279; range, 0-758,105,000), 18,846 HPV-16 DNA copies (mean, 2,759,939 ± 12,306,226; range, 0-104,660,000), and 4,124 HPV-16 DNA copies (mean, 1,016,650 ± 6,165,668; range, 0-69,720,000), respectively. The difference between the HPV-16 E6 assays was not significant ($P = 0.27$), whereas the difference between each HPV-16 E6–based assay and the HPV-16 L1 assay was statistically significant (Fig. 3; $P < 0.001$ for each comparison). The number of HPV-16 copies measured with the HPV-16 L1 assay was thus lower than the other assays. The number of specimens with >0,5 $\log_{10}$ HPV-16 DNA copies measured with the E6 PG assay compared with the HPV-16 L1 assay [78 (54.9%) of 142 samples] was significantly greater than the number of specimens with >0,5 $\log_{10}$ HPV-16 DNA copies measured with the HPV-16 L1 assay compared with the E6 PG assay [18 (12.7%) of 142 samples; $P < 0.001$]. The number of specimens with >0,5 $\log_{10}$ HPV-16 DNA copies measured with the HPV-16 E6 assay compared with the HPV-16 L1 assay [78 (54.9%) of 142 samples] was also significantly greater than the opposite [31 (21.8%) of 142 samples; $P < 0.001$].

HPV-16 DNA copy numbers obtained with each assay were also classified into tertile categories, and concordance between assays was evaluated (Table 3). Major differences (high-low results) were very rarely encountered (<1.5% of samples). Concordance rates reached 81.7% (116 of 142), 73.2% (104 of 142), and 72.5% (103 of 142) between HPV-16 E6 PG and E6 assays, E6 and L1 assays, and E6 PG and L1 assays, respectively.

We then evaluated if the correlation between assays was influenced by the cytologic status of participating women. A stronger correlation between assays was found in women with cytology smears suggestive of high-grade squamous intraepithelial lesions (ρ = 1.00, 0.96, and 0.96 between E6, L1 and E6 PG, and L1 and E6 assays, respectively) than in women with low-grade squamous intraepithelial lesions (ρ = 0.91, 0.86, and 0.82 between E6, L1 and E6 PG, and L1 and E6 assays, respectively) or in women with normal cytology results (ρ = 0.91, 0.82, and 0.85 between E6, L1 and E6 PG, and L1 and E6 assays, respectively). Coefficients of variation of real-time PCR assays have been shown to vary according to the quantity of measured DNA (31). We thus compared correlations between assays obtained in 76 samples with a high (≥5,000 HPV-16 L1 copies) and 66 samples with a low (<5,000 HPV-16 L1 copies) HPV-16 DNA copy number as measured by HPV-16 L1. Spearman's correlation coefficients between HPV-16 E6 or HPV-16 E6 PG assays and the HPV-16 L1 assay were significantly higher in samples with a greater number of HPV-16 L1 DNA copies (ρ = 0.75 and 0.76, respectively) compared with samples with a low number of HPV-16 DNA copies (ρ = 0.53 and 0.57, respectively; P = 0.03 and 0.04, respectively).

Table 1. Characteristics of 84 women tested for quantitation of HPV-16 DNA

Variable	No. (%) women	Variable value
Age		
Age categories (y)		
10-20	5 (6.0)	
20-29	35 (41.7)	
30-39	28 (33.3)	
40-49	13 (15.5)	
50-59	0 (0.0)	
60-70	1 (1.2)	
Unknown	2 (2.4)	
Average (y)		31.4 ± 8.5
Median (y)		30.4
Range (y)		18-63.3
HIV infection status		
HIV seronegative	18 (21.4)	
HIV seropositive	66 (78.6)	
CD4 cell count (cells/μL) measured for 66 women		
<200	21 (31.8)	
200-400	28 (42.4)	
>400	15 (22.7)	
CD4 not tested	2 (3.0)	
Average		304 ± 209
Median		262
Range		6-960
Ethnicity		
Caucasian	62 (73.8)	
African descent	14 (16.7)	
Asian	4 (4.8)	
First Nation	2 (2.4)	
Unknown	2 (2.4)	
Smoking		
Current smoker	40 (47.6)	
Not currently smoking	44 (52.4)	
HAART in 66 HIV-positive women		
HAART during HPV-16 infection	23 (34.9)	
No HAART during HPV-16 infection	43 (65.1)	
Age at first intercourse		
Age categories (y)		
10.1-12	2 (2.4)	
12.1-14	19 (22.6)	
14.1-16	26 (31.0)	
16.1-18	20 (23.8)	
18.1-20	9 (10.7)	
>20	3 (3.6)	
Unknown	5 (4.0)	
Average (y)		16.1 ± 2.3
Median (y)		16.0
Range (y)		12.0-22.0
Detection of HPV types other than 16		
Present	94 (65.3)	
Absent	50 (34.7)	
Cytology smear results at 144 visits		
Normal	77 (53.5)	
ASCUS	5 (3.5)	
LSIL	24 (16.7)	
HSIL	8 (5.5)	
Unsatisfactory	1 (0.7)	
Not done	29 (20.1)	
Colposcopy and histology results		
Normal	9 (10.7)	
CIN-1	12 (14.3)	
CIN-2	7 (8.3)	
CIN-3	3 (4.8)	
Not done	52 (61.9)	
HPV-16 LCR phylogenetic lineage		
European	57 (67.9)	
African 1	6 (7.1)	
African 2	3 (3.0)	
Asian American	3 (3.0)	
Not done	15 (17.9)	
HPV-16 E6 phylogenetic lineage		
European	56 (66.6)	
African	9 (10.2)	
Asian American	3 (3.0)	
Not done	16 (19.0)	
HPV-16 E6 variations		
E6 prototype	49 (58.3)	
E6 nonprototype	19 (22.6)	
Not done	16 (19.0)	

NOTE: Averages are means ± 1 SD.
Abbreviations: ASCUS, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; HAART, highly active antiretroviral therapy.

Correlation coefficients between HPV-16 E6 and E6 PG assays reached 0.92 and 0.84 in the latter sample groups, respectively. This difference was smaller than that seen with samples containing a higher HPV-16 copy number but remained significant ($P = 0.04$). The relationship between each assay was clearly linear with Pearson's coefficients ranging from 0.55 to 0.90 (all Ps < 0.001), with the highest correlation obtained between HPV-16 E6 assays ($r = 0.90$; 95% CI, 0.85-0.93).

Because of the association between race and HPV-16 polymorphism, the effect of viral polymorphism on the level of concordance in HPV-16 copy number estimates was investigated for the three real-time PCR assays. We first assessed if ethnicity was associated with HPV-16 LCR polymorphism, controlling for age and HIV status. Caucasian women were more likely than Black women [odds ratio (OR), 15.3; 95% CI, 3.0-78.9; $P = 0.001$] to be infected with a European HPV-16 variant than non-European variant. Older age (OR, 1.0; 95% CI, 0.9-1.2; $P = 0.51$) and being HIV seropositive (versus HIV-seronegative; OR, 2.2; 95% CI, 0.2-25.9; $P = 0.35$) were not significantly associated with having a European HPV-16 variant. The number of HPV-16 DNA copies measured by each real-time PCR assay was compared in samples from 62 Caucasian and 14 Black women. Spearman's correlation coefficient between these assays in specimens from Caucasian women ranged from 0.85 to 0.95. In Black women, the correlation coefficient was lower when the HPV-16 L1 was compared with HPV-16 E6 PG ($\rho = 0.71$; 95% CI, 0.29-0.91) or HPV-16 E6 ($\rho = 0.82$; 95% CI, 0.52-0.95) but not when HPV-16 E6 assays were compared ($\rho = 0.85$; 95% CI, 0.59-0.96). The difference of correlation coefficients between Caucasian and Black women was however not statistically significant ($P > 0.10$).

We investigated if the level of correlation between assays varied with HPV-16 polymorphism. One hundred one HPV-16 isolates belonging to the European phylogenetic branch generated an excellent correlation between assays: between HPV-16 E6 assays ($\rho = 0.96$; 95% CI, 0.94-0.98), HPV-16 L1 and HPV-16 E6 PG ($\rho = 0.86$; 95% CI, 0.78-0.92), or HPV-16 E6 ($\rho = 0.85$; 95% CI, 0.77-0.91). However, when isolates belonging to the African or Asian American phylogenetic groups were tested, an excellent correlation was found between HPV-16 E6 assays ($\rho = 0.93$; 95% CI, 0.77-0.98), whereas a lower correlation was shown between HPV-16 L1 and HPV-16 E6 PG ($\rho = 0.80$; 95% CI, 0.43-0.95) or HPV-16 E6 ($\rho = 0.76$; 95% CI, 0.33-0.93). This difference was however not statistically significant.

HPV-16 African variants carried three mismatches (at nucleotides 132, 143, and 145) in the region hybridizing with the HPV-16 E6 probe. A perfect homology was found between all HPV-16 E6 variants and primer or probe sequences used in the HPV-16 E6 PG assay (29, 49). Although as shown above the correlation between HPV-16 E6 assays did not seem to be affected by the three mismatches in probe sequence, the ratios of HPV-16 DNA copy numbers measured by HPV-16 E6 and HPV-16 E6 PG were significantly different

Table 2. HPV-16 DNA copies in 2 μL samples that tested negative in at least one real-time PCR test but positive in the MY09-MY11-HMB01 assay

No. samples	HPV-16 DNA copies		
	HPV-16 L1 assay	HPV-16 E6 assay	HPV-16 E6 PG assay
4	0 ± 0	0 ± 0	0 ± 0
3	0 ± 0	2,697 ± 3, 1,142 ± 3, 3,780 ± 4	1,638 ± 513, 188 ± 9, 1,454 ± 243
2	2,312 ± 8, 152 ± 197	0 ± 0	0 ± 0
2	6,180 ± 1,560, 82 ± 4	6,734 ± 124, 286 ± 13	0 ± 0
1	0 ± 0	0 ± 0	4 ± 2
1	0 ± 0	128 ± 2	21 ± 7

between 17 African isolates (median ratio, 0.53) from 9 women and 101 European isolates (median ratio, 1.31) from 56 women ($P = 0.002$).

HPV-16 viral loads were compared for a subset of 40 samples taking into account sample cellularity as measured by β-globin (for HPV-16 L1 and HPV-16 E6 assays) or ERV-3 (for HPV-16 E6 PG assay) real-time assays. HPV-16 viral loads varied from 0 to 2×10^9 HPV-16 DNA copies per microgram of cellular DNA. Because viral loads calculated from HPV-16 E6 and HPV-16 L1 assays were derived from the same estimations of cellular DNA, we did not compare results obtained with these two assays. Spearman's correlation coefficients reached 0.90 (95% CI, 0.82-0.95) between HPV-16 E6 PG and HPV-16 L1 assays and 0.86 (95% CI, 0.76-0.93) between HPV-16 E6 and HPV-16 L1 assays ($P < 0.0001$ for each correlation).

Discussion

This is the first assessment of the comparability of various real-time PCR assays in the quantitation of HPV-16 DNA and measurement of HPV-16 viral load. Interassay agreement is of importance in assessing the potential of HPV viral load as a biomarker for presence and progression of CIN, given the growing number of publications using real-time PCR assays for this purpose (8-10, 13, 15, 18, 30, 33, 34, 36, 50). The broad distribution of HPV-16 viral load values extending from a few copies up to 10^8 copies/2 μL sample permitted comparison of assay performance over a wide range of values. Furthermore, samples had been collected from women with varying cervical pathology, including 23 women with CIN and 37 women with abnormal cytology results. HIV-seropositive women represented the full spectrum of differing levels of immune suppression. Most HIV-infected women had CD4 counts between 200 and 400 cells/μL, a finding similar to that of the complete cohort (39). Several samples tested negative in at least one real-time PCR assay for HPV-16. Samples selected for this evaluation had initially tested positive for HPV-16 in a standard PCR protocol using MY09-MY11-HMB01 primers. The latter assay was applied on 5-μL lysate volume instead of the 2-μL volume used for real-time PCR assays. This difference could in part explain differences between initial testing and negative real-time PCR results.

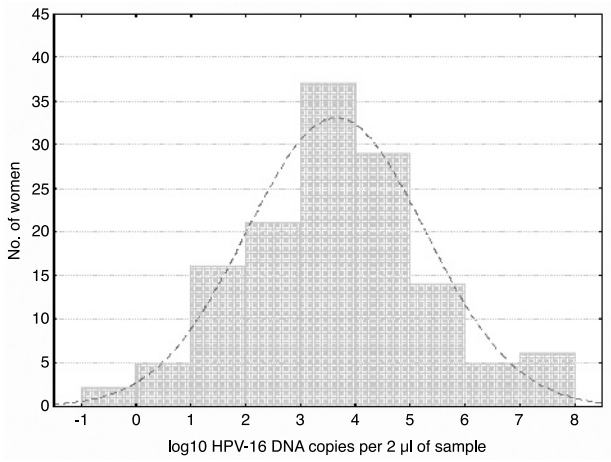

Figure 1. Distribution of HPV-16 DNA copies measured by HPV-16 L1 real-time PCR in 135 genital specimens. The amount of HPV-16 DNA in 2 μL sample was estimated with the HPV-16 L1 real-time PCR assay as described in Materials and Methods. Samples ($n = 9$) without HPV-16 DNA measured were excluded from the figure. The dotted line corresponds to expected normal values. The Kolmogorov-Smirnov test was not significant ($P > 0.2$), suggesting a normal distribution of data.

Our results suggest that there is excellent correlation between the quantity of HPV-16 DNA measured by three real-time PCR assays in processed genital specimens, particularly for the two HPV-16 E6 assays. However, the HPV-16 L1 assay measured significantly lower HPV-16 DNA copy numbers than did HPV-16 E6 assays of the same samples. Selection of samples with higher quantities of HPV-16 L1 DNA improved the level of correlation between assays. The level of correlation was lower between HPV-16 L1 and HPV-16 E6 assays than between the two HPV-16 E6 assays, although correlation was still good and statistically significant. These results are based on a small sample size, and additional confirmatory studies are needed. Underestimation of HPV-16 DNA quantity by the HPV-16 L1 assay could not be further explored in this work because we lacked data on HPV-16 L1 polymorphism.

The level of correlation between both HPV-16 E6 assays was very high, although different standard curves were used, two different laboratories did testing with different instruments, and specimens have been transported to one of the testing laboratories. Lower estimations of HPV-16 DNA quantity and viral load in some samples tested in laboratory B using the HPV-16 E6 PG assay compared with the HPV-16 E6 assay could be explained in part by DNA degradation or loss during transportation. In fact, two samples did not contain any human DNA when tested by laboratory B. HPV-16 viral load measures correlated more weakly in part due to the smaller number of samples compared but also due to the added variability introduced by evaluating cellular DNA content with two different assays in two different laboratories. Nevertheless, the level of correlation remained strong. If other studies confirm this level of correlation between real-time PCR assays, comparison between studies using these assays will be more meaningful.

The intra-assay reproducibility of two of the three real-time PCR assays has been described extensively in previous publications. The HPV-16 E6 PG test is highly reproducible over a wide linear dynamic range (33). It had an excellent intraclass correlation coefficient of 0.99 for HPV-16 quantitation, suggesting very low variability due to experimental error (33). The between-day variability of the latter assay for HPV-16 viral load has been estimated at 0.5% (33). A very high level of

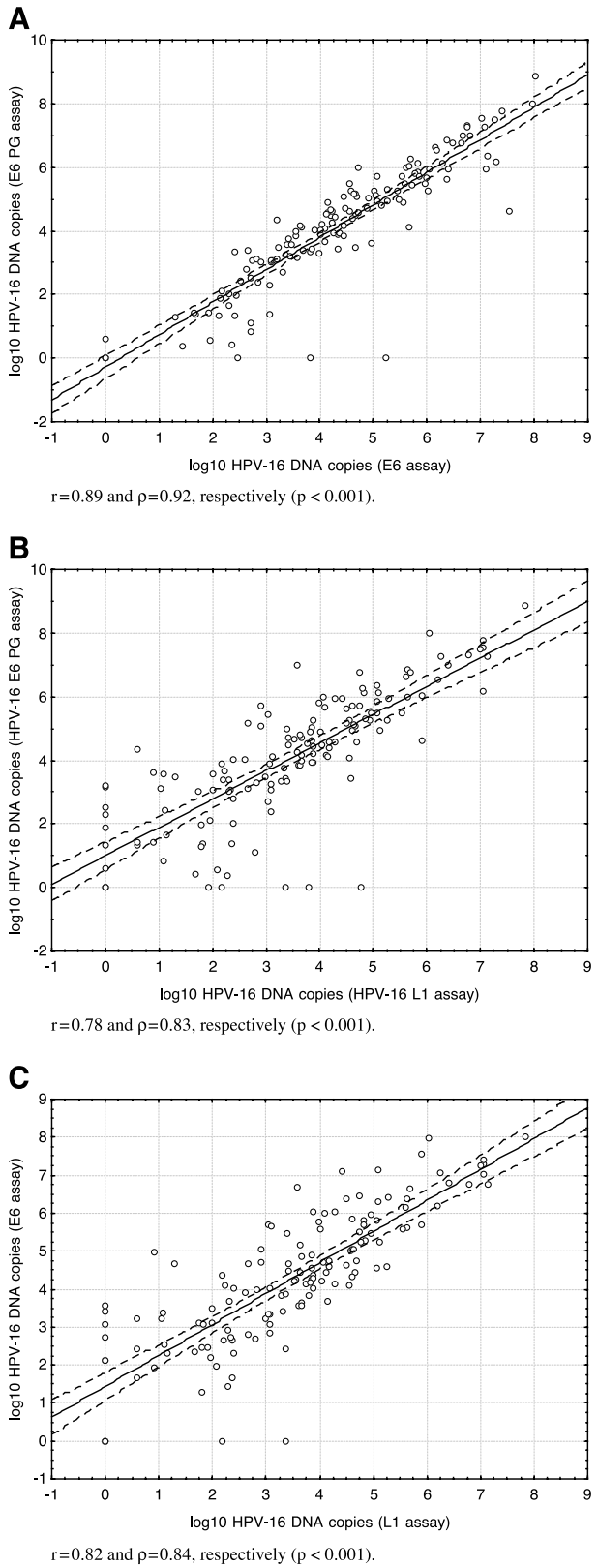

Figure 2. Comparison of number of HPV-16 DNA copies for 142 samples tested with HPV-16 E6, HPV-16 E6 PG, and HPV-16 L1 real-time PCR assays.

correlation was reached when results from two laboratories using the HPV-16 E6 PG assay were compared, with a Spearman correlation coefficient of 0.96. Inter-run coefficients of variations of the HPV-16 L1 assay (31) were higher in the presence of lower amounts of HPV-16 DNA but were as low as 12% in the presence of a higher number of HPV-16 copies (31). When samples with known quantities of HV-16 DNA were tested blindly with HPV-16 L1, the ratio of measured to expected viral load values reached 1.27 ± 0.32 (31). The intra-assay variability measured here between duplicates was within previously published ranges. The variability of HPV-16 E6 had not been described previously. The coefficient of variation measured with the HPV-16 E6 was comparable with that of the other two assays.

Similar evaluations on the reproducibility of HPV DNA quantitation have been conducted between Hybrid Capture 2 and real-time HPV-16 PCR assays (29, 30). When 40 samples were evaluated, correlation coefficients reached 0.87 for crude HPV-16 copy numbers and decreased to 0.74 when HPV-16 copies measured by real-time PCR were normalized for cell content (30). A larger evaluation, including 111 specimens, showed a relatively good crude agreement between Hybrid Capture 2 and real-time HPV-16 E6 PG assay, although with a much lower correlation than that found here between the HPV-16 E6 assays (29). One publication on the comparability of quantitative results using Hybrid Capture 2 reported interlaboratory correlation from 0.60 to 0.90 (51).

Considering the associations found previously between viral polymorphism, ethnicity, and viral load (28, 46, 52-54), we showed that ethnicity was associated with HPV-16 LCR polymorphism, controlling for age and HIV status. The correlation found between the HPV-16 E6–based assays was found to be dependent in part on HPV-16 polymorphism. This investigation revealed that signals obtained with the HPV-16 E6 assay for isolates carrying variations at three probe-binding sites tended to be lower compared with those obtained with the HPV-16 E6 PG assay using perfectly matched probes and primers. This difference was not shown when isolates without these variations in E6 were studied. We could not investigate if HPV-16 L1 polymorphism was responsible for the lower concordance between assays of non-European variants, due to primer or probe mismatch with HPV-16 sequences, because this information is not yet available in our cohort. However, L1 sequence data are available from a limited number of isolates and four sites of polymorphism have been described in the probe sequence (nucleotides 6862, 6864, 6865, and 6868) in European, Asian American, and African variants (46, 48, 55-58). Three sites of polymorphism have been described in the area hybridizing with the upper primer (nucleotides 6567, 6582, and 6576) and could impede amplification with the HPV-16 L1 assay (57, 59).

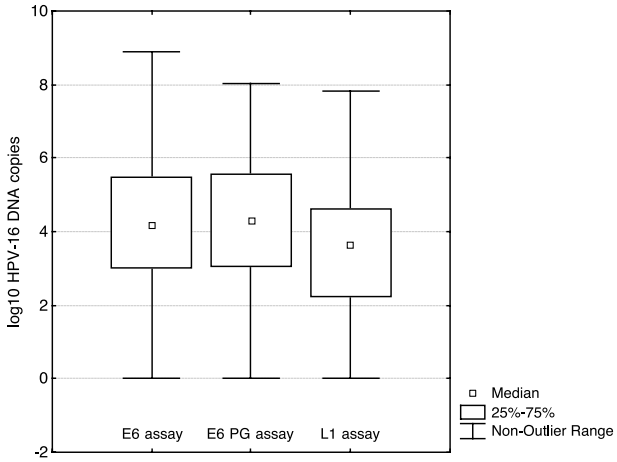

Figure 3. Comparison of the number of HPV-16 DNA copies in 142 genital samples measured by HPV-16 E6 PG, HPV-16 E6, and HPV-16 L1 assays.

Table 3. Comparison of HPV-16 viral copy categories obtained with three real-time PCR assays

No. HPV-16 E6 results*	No. HPV-16 E6 PG results		
	Low (<3.40)	Medium (3.40-5.00)	High (>5.00)
Low (<3.50)	42	5	0
Medium (3.50-5.00)	5	34	8
High (>5.00)	1	7	40
No. HPV-16 E6 results †	**No. HPV-16 L1 results**		
	Low (<2.70)	Medium (2.7-4.15)	High (>4.15)
Low (<3.50)	38	9	0
Medium (3.50-5.00)	9	28	12
High (>5.00)	0	10	38
No. HPV-16 E6 PG results ‡	**No. HPV-16 L1 results**		
	Low (<2.70)	Medium (2.70-4.15)	High (>4.15)
Low (<3.40)	37	10	1
Medium (3.40-5.00)	9	28	9
High (>5.00)	1	9	38

NOTE: HPV-16 values obtained with each real-time PCR assay were classified into tertile categories (low, medium, and high copy numbers) with ranges of viral loads in parentheses ($\log_{10}$ HPV-16 DNA copies).
*The κ value obtained for this table was 0.73 (95% CI, 0.61-0.84).
†The κ value obtained for this table was 0.60 (95% CI, 0.48-0.71).
‡The κ value obtained for this table was 0.59 (95% CI, 0.47-0.70).

When devising quantitative real-time PCR assays, great care should be taken to first characterize the genomic polymorphism of the HPV type targeted by the assay to ensure that differences in HPV DNA quantity or viral load are not due to impaired binding of probe or primers to HPV DNA rather than due to a truly lower HPV viral load. This information is critical considering that ethnicity is associated with HPV-16 polymorphism as well as with HPV viral load and both variables are associated with cervical cancer risk (46, 51, 60). HPV polymorphism could also explain lower levels of concordance related to ethnic origin of participants.

In conclusion, HPV-16 DNA copy numbers or viral loads measured with different real-time PCR assays correlated highly in this first comparison study of HPV-16 quantitation. Factors that could affect the level of correlation between assays include the number of HPV-16 DNA copies as well as viral polymorphism. Studies using the real-time PCR assays described here could be easily compared particularly if HPV-16 E6 assays are used for HPV-16 quantitation. Design of real-time HPV PCR assays requires knowledge of primer and probe design as well as polymorphism of the region amplified in the HPV genome. Samples analyzed here were obtained from women infected or at high risk for HIV. Further studies should be undertaken in older women, in women at lower risk for sexually transmitted infections, and in women with high-grade cervical disease. Analysis of HPV-16 L1 polymorphism could shed some light on the reasons for lower reactivity in this assay.

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High Level of Correlation of Human Papillomavirus-16 DNA Viral Load Estimates Generated by Three Real-time PCR Assays Applied on Genital Specimens

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