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Comparison of Three Different PCR Methods for Quantifying Human Papillomavirus Type 16 DNA in Cervical Scrape Specimens

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We compared real-time LightCycler and TaqMan assays and the GP5+/6+ PCR/enzyme immunoassay (EIA) to assess the human papillomavirus type 16 (HPV16) load in cervical scrape specimens. Both real-time PCR assays determined the HPV16 load in scrape specimens similarly. The level of agreement between these assays and the GP5+/6+ PCR/EIA was low ($P = 0.004$), suggesting that the latter method is not suited for quantifying HPV16 DNA.

It is now well established that a persistent infection with a high-risk human papillomavirus (hr-HPV) type is a necessary cause of cervical cancer (1, 7, 14). In addition, several studies have shown that an increased human papillomavirus (HPV) DNA load within a cervical smear specimen is associated with an increased risk of cervical intraepithelial neoplasia grade 3 (CIN3) and cervical carcinomas (3, 6, 11, 13). Therefore, viral load assessments as follow-ups of consensus hr-HPV tests may have implications for the identification of women among those with hr-HPV infections that have the highest risk of CIN3 lesions or cervical carcinomas (10). However, data on the possible clinical value of viral load are not consistent, which in part may reflect the use of different quantification assays (4, 8, 9).

Here, we compared a newly developed real-time PCR assay based on fluorescence resonance energy transfer (LightCycler; see Table 1) with a previously described real-time PCR assay based on fluorigenic 5′ nuclease chemistry (TaqMan) (13) and the semiquantitative consensus HPV GP5+/6+ PCR enzyme immunoassay (EIA) (12) for their abilities to assess the HPV16 load in cervical scrapings as a follow-up of GP5+/6+ PCR typing. To that end, a total of 109 cervical scrapings were randomly selected from a population-based screening study among scrape specimens that were HPV16 positive by GP5+/6+ PCR genotyping (12). These comprised 54 samples classified as normal and 55 classified as borderline or mild dyskaryosis (BMD). BMD equals atypical squamous cells (ASC) of undetermined significance/ASC–high-grade squamous intraepithelial lesion/ASC–low-grade squamous intraepithelial lesion, according to the Bethesda 2001 classification (2). The scrape specimens comprised 80 cases with single infections and 29 cases with multiple infections including HPV16. Thirteen randomly chosen GP5+/6+ PCR-negative cervical scrapings were also subjected to real-time PCR assays.

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Real-time PCR was performed on 100 ng target DNA using reagents the same as those described by Stevens et al. (10), except that 3.5 mM $MgCl_2$ was used for HPV16. Details of primers, probes, and cycling determinants are given in Table 1. For HPV16 quantification, standard curves of 10-fold dilutions of pHPV16 plasmid DNA (ranging from 10 to 10^5 fg) spiked in 100 ng human placental DNA were used, whereas those of the β-globin gene contained only human placental DNA (ranging from 3 pg to 30 ng). Samples were tested in duplicate, and the values obtained were averaged. The real-time PCR assays determined the HPV16 load normalized per cell and per scrape specimen, whereas the GP5+/6+ PCR/EIA assayed the load per scrape specimen expressed as EIA optical density (OD) values after a 1-hour substrate incubation (5).

The median HPV16 loads per cell of the 109 cervical samples determined by the LightCycler and TaqMan assays were 4.0 copies/cell (range, 0.0 to 137.4) and 6.7 copies/cell (range, 0.0 to 346.2), respectively. The HPV16 copies/cell values measured by TaqMan were on average 1.6 times higher (95% confidence interval [CI], 1.3 to 2.1) than those measured by LightCycler. The correlation between both assays for determining HPV16 copies/cell was strong (Spearman $\rho = 0.84$; Fig. 1A) and their agreements were moderate (kappa value calculated after categorization of the variables in tertiles, 0.59). Correlation and agreement were not significantly influenced by the level of dyskaryosis (normal cytology or BMD) or the number of HPV types present in a scrape specimen. None of the 13 GP5+/6+ PCR-negative cervical scrape specimens revealed any HPV16 signal in both LightCycler and TaqMan assays, whereas all revealed β-globin gene PCR signals with both assays within the same range as that of the GP5+/6+ PCR-positive scrapings.

Subsequently, the semiquantitative GP5+/6+ PCR/EIA was used to determine the HPV16 load per cervical scrape specimen. The median EIA OD value of the GP5+/6+ PCR after 1-hour substrate incubation was 1.17 (range, 0.07 to 1.67). The median numbers of calculated HPV16 copies/scrape specimen were 5.1×10^6 (range, 3.3×10^4 to 2.5×10^9) and 8.1×10^6

TABLE 1. Primers, probes, and PCR specifications for the quantification of HPV16 and β-globin targets

Assay (length of PCR amplicon [bp])	Sequence (5′ → 3′)[a]	Position in genome[b]	Cycling conditions[c]
LightCycler HPV16 (158)	fw: GAGGAGGAGGATGAAATAGATGGT	658–681	den: 95°C/3 s
	re: GCCCATTAACAGGTCTTCCAA	816–796	ann: 56°C/15 s
	dp: ACAAAAGGTTACAATATTGTAATGGGCTCT	735–706	elon: 72°C/9 s
	ap: CCGGTTCTGCTTGTCCAGCTGG	703–682	
LightCycler β-globin (196)	fw: GAGCCATCTATTGCTTACATTTGC	62122–62145	den: 95°C/3 s
	re: TTGGTCTCCTTAAACCTGTCTTGT	62318–62295	ann: 55°C/15 s
	dp: CCAGGGCCTCACCACCAACTTC	62275–62254	elon: 72°C/10 s
	ap: CCACGTTCACCTTGCCCCACAG	62251–62230	
TaqMan HPV16 (139)	fw: CAGATACACAGCGGCTGGTTT	5914–5934	13
	re: TGCATTTGCTGCATAAGCACTA	6053–6032	
	Probe: TGACCACGACCTACCTCAACACCTACACAGG	5936–5966	
TaqMan β-globin (152)	fw: GAGCCATCTATTGCTTACATTTGC	403–426	13
	re: TTGGTCTCCTTAAACCTGTCTTGT	555–532	
	Probe: TCTACCCTTGGACCCAGAGGTTCTTTGAGT	471–501	
GP5+/6+ PCR (141)	fw: TTTGTTACTGTGGTAGATACTAC	6624–6646	12
	re: CTTTTTATTGACATTTAGTATAAG	6741–6764	
	Probe: GTCATTATGTGCTGCCATATCTACTTCAGA	6662–6691	

[a] fw, forward primer; re, reverse primer; dp, donor probe (3′ end labeled with fluorescein); ap, acceptor probe (3′ end phosphorylated, HPV16 5′ end labeled with LightCycler-Red-640 and β-globin 5′ end labeled with LightCycler-Red-705). TaqMan probes are at the 5′ end 6-carboxyfluorescein and at the 3′ end 6-carboxytetramethylrhodamine labeled; the EIA probe was 5′ end digoxigenin labeled.

[b] GenBank accession numbers for HPV16 and β-globin are K02718 and U01317, respectively.

[c] den, denaturation temperature and time; ann, annealing temperature and time; elon, elongation temperature and time. Cycling conditions for the final three assays were as described previously by van Duin et al. (13) or by van den Brule et al. (12), as indicated.

(range, 8.0×10^4 to 4.0×10^9) by LightCycler and TaqMan, respectively. The TaqMan assay determined the number of HPV16 copies/scrape specimen to be on average 1.7 times (95% CI, 1.3 to 2.2) higher than the LightCycler assay did. Conversely, the median amount of cells per scrape specimen as calculated by both techniques did not differ (mean of β-globin ratios, 1.00; 95% CI, 1.00 to 1.01). Correlation and agreement were strong (Spearman $\rho = 0.87$) and good (kappa value = 0.64), respectively, between the two real-time PCR assays in determining the HPV16 load/scrape specimen (Fig. 1B). These values decreased when comparing either of the two real-time PCR assays with the semiquantitative GP5+/6+ PCR/EIA (Fig. 1C and D). Correlation coefficients were 0.81 and 0.77 and kappa values were 0.53 and 0.50 for GP5+/6+ PCR/EIA values versus LightCycler and TaqMan values, respectively. The number of HPV types present in a scrape specimen or the degree of dyskaryosis did not significantly influence the correlations. By means of a regression model for standardized values, we tested for differential effects of TaqMan and GP5+/6+ PCR/EIA on LightCycler values and found a significantly higher coherence between the results for the different real-time PCR assays than between the results for GP5+/6+ PCR/EIA and the LightCycler assay ($P = 0.004$).

The fact that TaqMan scored the HPV16 load overall higher by a factor 1.6 to 1.7 than the LightCycler assay did could be due to many factors. However, variables such as HPV16 primer composition and the use of nonlinearized plasmids in the standard curve are most likely to be the underlying cause. Nevertheless, this difference was consistent throughout the whole detection range, making data obtained with these two methods still comparable when using a correction factor. Moreover, data collected so far support the concept that there exist differences in viral load values of several magnitudes between clinically relevant and irrelevant HPV infections (10, 13). Therefore, it is unlikely that a factor difference of 1.6 to 1.7 in HPV16 load measured by the two real-time assays would be of clinical relevance.

It should be realized that this comparative analysis was performed on a random, cross-sectional series of smear specimens with normal cytology or BMD. As a high viral load may be an important determinant of CIN3 lesions or cervical carcinomas (reviewed in reference 9), the choice of these samples could potentially have led to studying a range of viral copy numbers that fall beyond the level that would represent high-grade cervical lesions. However, preliminary HPV16, -18, -31, and -33 viral load analysis by LightCycler on cervical smear specimens of women having underlying high-grade CIN lesions revealed ranges of 9.2×10^4 to 5.3×10^8, 7.6×10^4 to 5.1×10^8, 2.4×10^4 to 9.4×10^8, and 2.0×10^6 to 8.5×10^8, respectively (C. J. Hogenwoning et al., unpublished results). These ranges correspond to the range of HPV16 copies/scrape specimen found by the LightCycler assay (i.e., 3.3×10^4 to 2.5×10^9) analyzed in this comparative study on smear specimens from women with as-yet-unknown clinical outcomes.

In summary, we demonstrated that the TaqMan and the LightCycler real-time PCR technologies produce similar results for the quantification of HPV16 DNA in cervical scrape specimens and that both are therefore useful in the detection of viral load in clinically relevant HPV infections. However, the GP5+/6+ PCR/EIA assay was not adequate for a precise assessment of the HPV load. Our data can be of value for future comparisons of studies involving viral

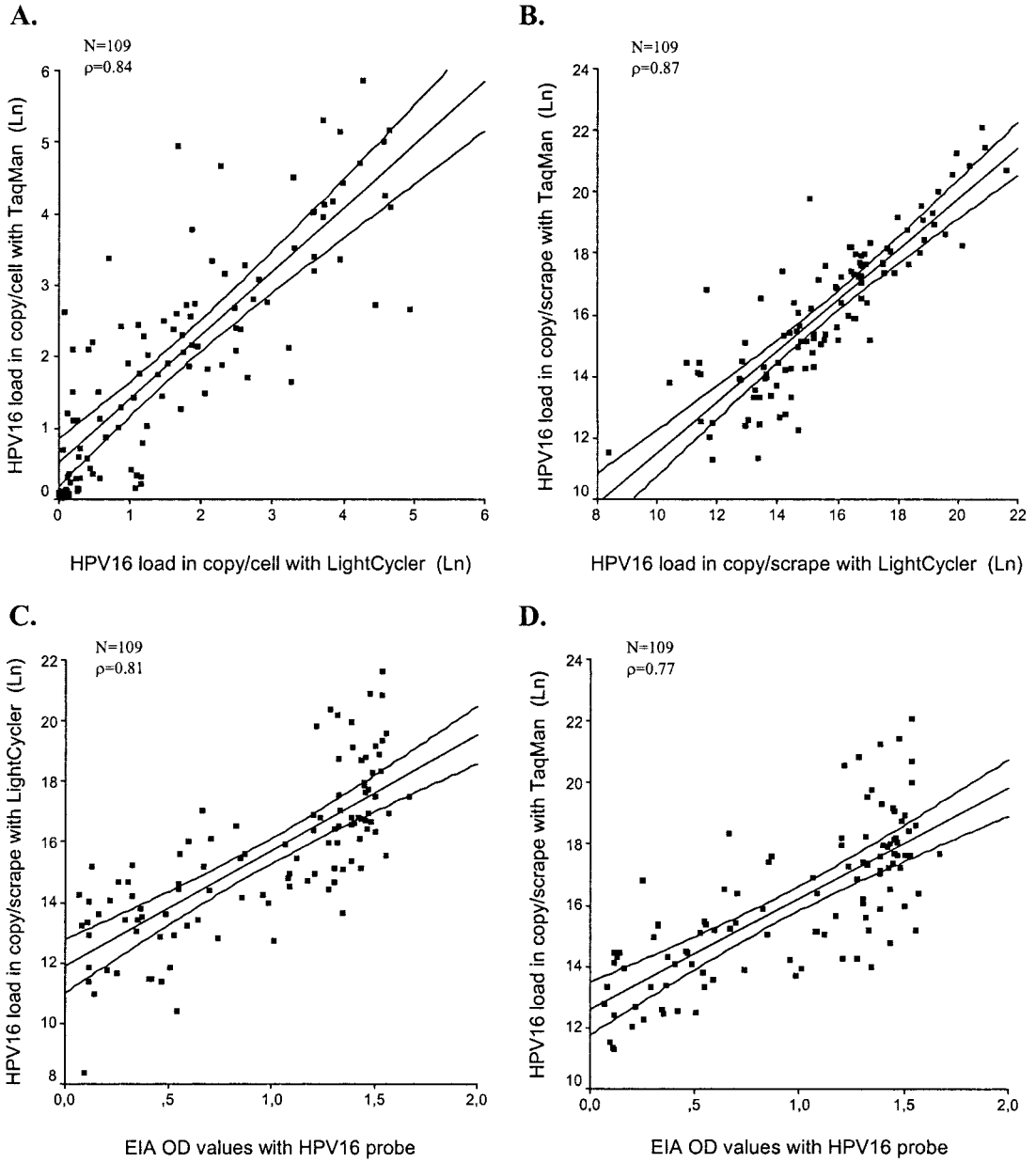

FIG. 1. Correlation of HPV16 DNA loads as determined by different assays. (A) Correlation of TaqMan (*y* axis) and LightCycler (*x* axis) assays stratified for cellular input. (B) Correlation of TaqMan (*y* axis) and LightCycler (*x* axis) assays in assessing HPV16 load per scrape specimen. (C) GP5+/6+ PCR/EIA OD values (*x* axis) plotted against the LightCycler HPV16 load/scrape specimen (*y* axis). (D) GP5+/6+ PCR/EIA OD values (*x* axis) plotted against the TaqMan HPV16 load/scrape specimen (*y* axis). All values determined by real-time PCR are ln normalized in the plots. The inner line in each plot represents the regression line, whereas the outer lines indicate the 99% confidence interval of the regression line. Spearman correlation coefficients (ρ) are indicated in each plot.

load assessment in relation to the risk of CIN3 lesions or cervical carcinomas. In our setting, one such study is currently in progress.

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