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Development and interlaboratory agreement of real-time PCR for HPV16 quantification in liquid-based cervical samples

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ABSTRACT

High risk HPV infection is the necessary cause for the development of precancerous and cancerous lesions of the cervix. Among HPV, HPV16 represents the most carcinogenic type. Since the determination of HPV16 DNA load could be clinically useful, we assessed quantitative real-time PCR targeting E6HPV16 and albumin genes on two different platforms. Series of SiHa cells diluted in PreservCyt were used to assess repeatability and reproducibility of two in-house real-time PCR techniques run in two different laboratories to determine HPV16 load. Furthermore, 97 HPV16 positive cervical samples were evaluated to estimate inter-center variability using Bland-Alman plots. As a whole, both techniques presented coefficients of variation for HPV16 load measurement similar to those established for other virus quantification with commercial kits. Moreover, the two real-time PCR techniques showed a very good agreement for HPV16 load calculation. Finally, we emphasize that robust HPV16 DNA quantification requires normalization of viral load by the cell number.

1. Introduction

High risk human papillomaviruses (hrHPV) have been recognized as the etiologic agents for cervical cancer [1]. Among the 40 genotypes known to infect the anogenital mucosa, HPV16 is the most prevalent genotype worldwide [2–4]. HPV16 is also classified as the most carcinogenic virus to humans by IARC. This is likely due to its high potency of persistence of 12 months or more, the necessary cause for pre-malignant and malignant lesion development [5–7].

Large randomized clinical trials have demonstrated the excellent performance of hrHPV testing, in combination or not with cytology, for the detection of precancerous lesions and cancer of the cervix [8–12]. Indeed, hrHPV DNA testing improves the screening sensitivity. However, because, most infections will be cleared within few years (90% at 2–3 years) [13], hrHPV testing presents a low specificity and positive predictive value. Therefore, there is a need to assess novel biomarkers to efficiently manage women who are tested positive for an hrHPV. One available option for triaging hrHPV infected women consists in HPV16/HPV18 genotyping because infection by one of those types is associated with a high risk of CIN2/3+ lesion [14].

We and others have developed real-time PCR-based technique to determine whether type specific HPV viral load could serve as diagnostic and/or prognostic marker to improve HPV specificity. Several studies reported that high HPV16 DNA load was associated with prevalent high grade lesions or cancers [15–24]. Moreover, a cutoff value of 22,000 copies/10^3 cells permits to identify among HPV16 infected women those with a high grade lesion with a specificity of 90% [20]. Furthermore, HPV16 load may represent a risk factor for persisting infection [25–28] or progression toward a high grade cervical lesion [29,30]. In a retrospective longitudinal study, a HPV16 viral load of 200 copies/10^3 cells was shown to represent a relevant cutoff above which women with no or mild abnormalities of the cervix present a high risk to develop an incident CIN2/3 at 18 months [29]. An elegant nested case-control study revealed that HPV16 load of newly acquired infection and variations in HPV16 load was associated with an increased risk of CIN3 [31]. Thus quantification of HPV16 DNA load

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Table 1
Primers and probes used for HPV16 load quantification.

Target		Sequence	Concentration	
			Center 1	Center 2
ALB[a]	Forward primer	5′-GCTGTCATCTCTTGTGGGCTGT – 3′	300 nM	500 nM
	Reverse primer	5′-ACTCATGGGAGCTGCTGGTTC – 3′	300 nM	500 nM
	Probe	5′-FLUOR[b]-CCTGTCATGCCCACACAAATCTCTCC-BHQ – 1–3′	100 nM	250 nM
E6 HPV16	Forward primer	5′-GAGAACTGCAATGTTTCAGGACC – 3′	100 nM	500 nM
	Reverse primer	5′-TGTATAGTTGTTTGCAGCTCTGTGC – 3	100 nM	500 nM
	Probe	5′ – 6-FAM-CAGGAGCGACCCAGAAAGTTACCACAGTT-BHQ – 1–3′	100 nM	250 nM

[a] Albumin gene.
[b] Donor fluorophore (FLUOR) was FAM in center 1 and HEX in center 2.

might allow the identification of women with prevalent lesions or at increased risk of developing precancerous and cancerous cervical lesions.

Real-time PCR permits a rapid and accurate quantification of molecular targets with special usefulness as diagnostic, prognostic or theranostic applications. Nevertheless, the standardization of procedures is a prerequisite to ensure the reliability of new molecular tests. This is especially true if protocols have to be shared by different laboratories and when local practices (specific consumables, equipment, DNA extraction, cell collection medium) may directly influence the results.

The aim of this study was to assess protocols to quantify HPV16 genome on two real-time PCR platforms, in two different laboratories referred to as center 1 and center 2.

2. Material and methods

2.1. Study design

Evaluation of HPV16 real-time PCR protocols was conducted in two steps. The first step aimed at assessing in each center percentage coefficient of variations (%CV) for repeatability (intra-assay variability) and reproducibility (inter-laboratory variability) experiments for HPV16 viral load measurement from SiHa cell dilutions. The second step aimed at measuring agreement between laboratories using a pragmatic approach based on the same series of clinical samples analyzed in both centers. Bland-Altman plots was used to test the agreement between the two centers for E6 and albumin gene (*ALB*) copy numbers and HPV16 viral load quantification.

2.2. SiHa cell preparation

SiHa cells, harboring 1–2 integrated HPV16 genomes, were cultivated in center 1 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum at 37 °C in a humidified incubator and in the presence of 5% of CO2. SiHa cell dilutions were prepared in order to obtain a low (10^3), medium (10^5) and high (10^7) number of SiHa cells suspended in 20 mL of PreservCyt™. Dilutions were centrally prepared in the center 1 and an aliquot was sent to the center 2. DNA extraction was carried out from 2 mL of SiHa cell dilutions. In the center 1, after centrifugation the cell pellet was lysed using proteinase K at 56 °C for 3 h. Then, DNA extraction was performed with the EZ1 DNA tissue kit and the BioRobot EZ1 (Qiagen Inc., Courtaboeuf, France) according to the manufacturer's instructions. In the center 2, after centrifugation, the cell pellet was diluted in 400 µL of AL buffer (Qiagen) with 40 µL of proteinase K and incubated at 56 °C overnight. The lysates were then filtered through the column provided in the QIAamp DNA mini kit (Qiagen) and DNA was eluted with 80 µL of molecular grade water.

2.3. Repeatability and reproducibility assessment

Repeatability and reproducibility were assessed in both the center 1 and the center 2. For repeatability, the 3 dilutions of SiHa cells were tested 12 times for *ALB* and E6 copy number and for HPV16 viral load quantification in the same run and under the same conditions. For reproducibility, the 3 dilutions of SiHa cells were tested in duplicate in 6 independent experiments. Percentage coefficient of variations were calculated for each parameters: *ALB* and E6 copy number and HPV16 viral load.

2.4. Clinical specimens

Cervical samples were collected in PreservCyt™ Medium (ThinPrep, Cytyc Corp, Marlborough, Mass, USA) from 97 women who underwent their routine cervical screening in the Department of Obstetrics and Gynecology from the University Hospital of Reims. All specimens were tested positive with the hc2 assay (Qiagen) and confirmed to be HPV16 positive with the Linear Array HPV genotyping test (Roche Diagnostics, Meylan, France). DNA was isolated from residual liquid-based cytology material (4 mL) using the EZ1 DNA tissue kit and the BioRobot EZ1 (Qiagen) and eluted in 200 µL buffer. An aliquot of 35 µL of DNA was prepared from each sample and sent to the center 2. The 97 samples were run in duplicate to determine *ALB* and E6HPV16 copy numbers and HPV16 viral load.

2.5. Real-time PCR for ALB and E6HPV16 gene quantification

Same primers and probes (Eurogentec, Seraing, Belgium) were used in both centers (Table 1). PCR reactions were conducted with specific consumables dedicated to the LC480 thermocycler (Roche Diagnostics, Meylan, France) in the center 1 and to the ABI7500 thermocycler (Applied Biosystems, Courtaboeuf, France) in the center 2.

For each sample, the cell number was estimated using a real-time PCR targeting the albumin gene. The reference human genomic DNA provided by Roche Diagnostics (Meylan, France) was serially diluted and run in parallel with the DNA from cervical samples to build the standard curve as previously described [32].

The number of HPV16 genome copies was determined using a real-time PCR targeting the E6HPV16 gene. Standard curves were obtained by the amplification of a dilution series of 2.10^6 copies to 200 copies of an HPV16 plasmid prepared in the center 2 and then distributed to the center 1.

In the center 1, thermocycling conditions were 1 × 10 min at 95 °C followed by 45 × (15 s at 95 °C and 1 min at 65 °C) then 1 × 30 s at 40 °C for *ALB* and E6 at the exception of the temperature of extension set at 60 °C for E6HPV16. In the center 2, thermocycling conditions were 1 × 2 min at 50 °C followed by 1 × 10 min at 95 °C and 40 × (15 s at 95 °C and 1 min at 60 °C) for both *ALB* and E6HPV16. A non-contamination control (molecular grade water) was used in duplicate in each run. The second derivative method was used to calculate Cp values. HPV16 load was calculated and normalized with respect to the cell

Table 2
Repeatability (Repeat.) and reproducibility (Reprod.) were determined with samples harboring a low number of SiHa cells (10^3 SiHa cells/20 mL of PreservCyt corresponding to 10 *ALB* eg or 10 HPV16 eg in the reaction tube), a medium number of SiHa cells (10^5 SiHa cells/20 mL of PreservCyt corresponding to 10^3 *ALB* eg or 10^3 HPV16 eg in the reaction tube) and a high number of SiHa cells (10^7 SiHa cells/20 mL of PreservCyt corresponding to 10^5 *ALB* eg or 10^5 HPV16 eg in the reaction tube). Mean Nb: mean number of eg found in the reaction tube. Normalized viral load corresponds to the mean viral load determined from each replicate. *ALB*: albumin gene, CV: coefficient of variation, eg: equivalent genome, na: not available due to outlier values.

	ALB		HPV16 E6		Normalized HPV16 load		Corrected HPV16 load[a]
	Mean Nb (log)	CV%	Mean Nb (log)	CV%	eg/cell	CV%	eg/cell
Repeat. center 1							
Low	1.0	14%	1.9	8.9%	19.3	25%	5
Medium	2.9	8.0%	3.9	4.8%	20.6	17%	6
High	4.9	1.4%	5.8	1.3%	15.5	7%	4
Reprod. center 1							
Low	na	na	1.1	67%	15.5	62%	6
Medium	2.1	30%	3.1	17%	24.4	42%	6
High	4.6	5.9%	5.6	3.7%	18.6	37%	5
Repeat. center 2							
Low	0.4	43%	0.9	13%	7.6	46%	2.5
Medium	2.3	2.0%	2.9	1.7%	9.6	5%	3
High	4.2	0.5%	5.0	1.0%	13	7%	4.2
Reprod. center 2							
Low	0.7	30%	0.9	16%	3.2	46%	1
Medium	2.5	4.6%	2.9	2.5%	6	23%	1.9
High	4.4	1.0%	5.0	1.0%	7.6	8%	2.5

[a] HPV16 viral load was corrected with the internal calibrator: SiHa cells, 2 HPV16 eg per cell for the center 1; CaSki cells, 600 HPV16 eg per cell for the center 2.

number considering that each cell harbors 2 albumin gene copies. Normalized HPV16 load was then expressed as a number of HPV16 equivalent genome per cell (eg/cell). The HPV16 real-time PCR proved to be specific as we previously published no cross reactivity with other hrHPV [33]

As a further refinement of the HPV16 viral load calculation an internal calibrator, consisting in SiHa cell DNA (2 HPV16 eg/cell) for the center 1 and Ca Ski cell DNA (600 HPV16eg/cell) for the center 2, was introduced in each run. A corrected HPV16 load was then calculated.

2.6. Data analysis

HPV16 copy number and *ALB* copy number were log10 transformed for convenience because a large dynamic range was observed for these values. Conversely, non-log transformed values were kept for HPV16 load as it was normalized with the cell number. Agreement between the two centers was determined using the Bland-Altman method [34]. For each sample, the difference between respective values (Y axis) from each experiment was plotted against their means (X axis). The assumption of normality of the differences could not be considerate as valid. A non-parametric approach was used to compare methods [35]. The limits of agreement (LoA) were estimated with the 2.5th and 97.5th percentiles of the differences; the average bias was estimated by the median of the differences. Statistical analyses were performed using the MEDCALC and Systat softwares.

3. Results and discussion

3.1. Repeatability and reproducibility assessment

Repeatability and reproducibility data, obtained from 12 measurements, are presented in Table 2. The absolute quantification of *ALB* and E6 genes showed variable results across the different SiHa cell dilutions (Table 2). The albumin gene copy number was inferior to the theoretical target in the center 2. In contrast, the E6 gene copy number tended to be overestimated in the center 1, while it reached the targeted values in the center 2.

As a whole, this probably explains why the experimental viral loads, that ranged from 15.5 to 24.4 HPV16 eg/cell for center 1 and from 3.2 to 9.6 for center 2, were overestimated compared to the expected values of 1–2 HPV16 eg/cell. After applying a correction factor based on the internal calibrator, the corrected HPV16 load values varied from 4 to 6 HPV16 eg/cell for the center 1 and from 1 to 4 HPV16eg/cell for the center 2. Thus, the use of an internal calibrator improved the precision of the quantitative measurement of HPV16 load.

As for the quantification of albumin and E6 genes, the coefficients of variation (CV) ranged from 0.5% to 43% for repeatability and from 1% to 67% for reproducibility. After normalization, the CV for HPV16 DNA load ranged from 7% to 46% for repeatability and from 8% to 62% for reproducibility. As expected the highest CV were calculated for samples with the lowest copy number of albumin genes and HPV16 genomes. Furthermore, the coefficients of variation were higher in the reproducibility than in the repeatability experiments. This was also expected since reproducibility experiments measure more sources for variation (e.g. caused by the operator himself and interaction between the operator and the sample) than repeatability experiments that evaluates only variation due to the measuring device. This is in line with the observations by Gravitt et al. who studied reproducibility of HPV16 and HPV18 DNA load quantification [36]. At this time, there is no recommendation concerning expected CV% values for HPV16 load measurement to make comparison. Generally, commercial kits dedicated to the quantification of other viruses recommend a maximum CV% of 15–20% (e.g. Abbott real time HIV, Abbott real time HCV or Abbott real time HBV). Nevertheless, a study conducted with specimens harboring low HIV viral loads reported CV% ranging from 26% to 59% with completely automated systems [37]. Thus, it can be assumed that the two in-house real-time PCR techniques used in this study to quantify HPV16 viral load fulfill quality criteria in terms of repeatability and reproducibility.

3.2. Agreement between centers is increased by HPV16 load normalization

The median of the differences for *ALB* and E6 quantification from clinical samples were −0.16 log copies/µL (95% LoA [-0.39; −0.03]) and −0.27 log copies/µL (95% LoA [-0.83; −0.11]) respectively (Fig. 1A). These medians of differences were very close to zero but LoA, within which 95% of differences between measurements by the two centers are expected to lie, did not include 0. This probably comes from a few clinical samples with discrepant results. The retrospective analysis of clinical/virological annotations of samples with discrepant

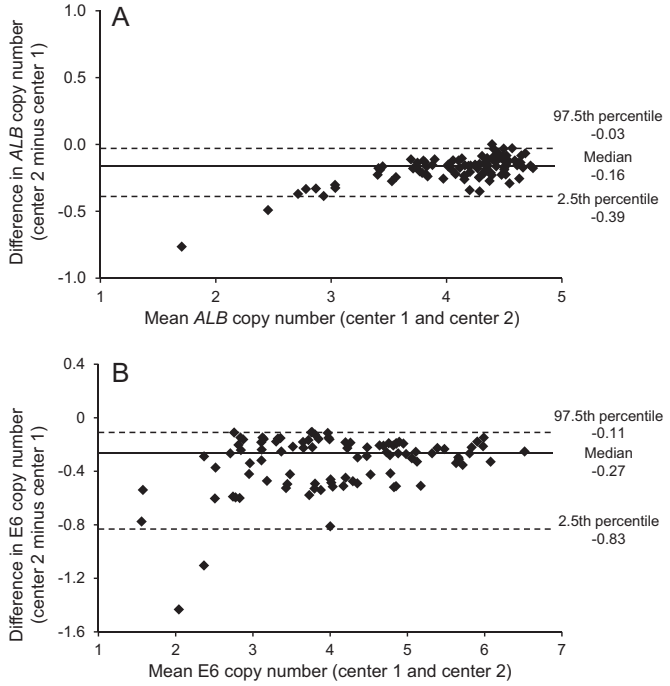

Fig. 1. Bland Altman plots for *ALB* (A) and E6 (B) copy number in center 1 compared to center 2. The median of the differences and the 95% limits of agreement (2.5th and 97.5th percentiles) are indicated as solid and dashed lines respectively. *ALB*: albumin gene.

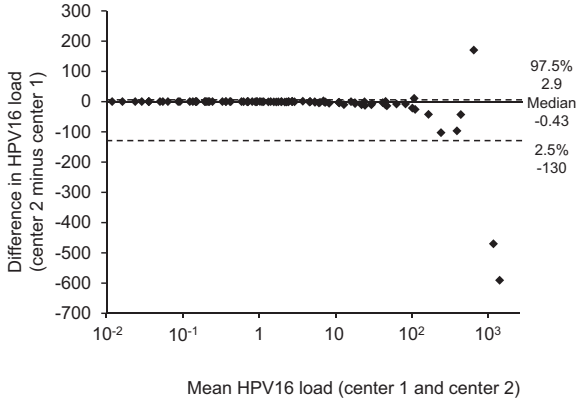

Fig. 2. Bland Altman plots for normalized HPV16 DNA load (eg/cell) in center 1 compared to center 2. The median of the differences and the 95% limits of agreement (2.5th and 97.5th percentiles) are indicated as solid and dashed lines respectively.

results did not reveal any major sampling bias. Discrepancies were unlikely due to the pre-analytical step because DNA extraction was centralized and automated. However, DNA quality may have been altered due to multiple freezing/thawing cycles during the experimental protocol. Because low amount of DNA was available, these samples could not be retested. Thus a good agreement for the quantification of E6HPV16 and albumin gene copy numbers was achieved between the two centers. It is worthy to note that the differences never exceeded 1 log copy. Such agreement is consistent with those observed, for example, for HIV-1 plasma viral load determination with commercial assays [38].

When E6HPV16 copy number was normalized by the number of cells, agreement between the two centers became excellent (Fig. 2). Indeed, the median of the differences between HPV16 load was −0.43 copies/µL (95% LoA [-130; 2.9]). This median was close to zero and the 95% LoA included 0. This result emphasizes that HPV16 DNA load should be normalized with the number of cells in order to compensate for the overall process variability. Another important point relies on the use of the same series of HPV16 plasmid standards for E6HPV16 quantification in both centers. Indeed, preliminary experiments conducted with HPV16 standards prepared in each center showed a low agreement for HPV16 load calculation (not shown). Thus it can be recommended to use well calibrated HPV16 standards to achieve comparable HPV16 load in laboratories. Limitations in this study rely first on the fact that agreement between the two centers was evaluated from DNA and not from cervical samples. Thus it cannot be excluded that the assays performed less well if centers have started from cell suspensions. It is noteworthy that the work was conducted on residual liquid-based cytology samples and the scarcity of some residual materials precluded to split samples to proceed to two DNA extractions. Second, no internal calibrator was run in parallel with each series of clinical samples. It would have certainly improved the accuracy of the measurement as shown for SiHa cell suspensions and increased inter-laboratory agreement.

4. Conclusions

Several studies have shown that HPV16 load could be a relevant biomarker to identify women with prevalent lesions or at risk of incident lesions of the uterine cervix and some authors have proposed thresholds to triage women at risk [20,29,30,39]. Nevertheless, clear thresholds could not be highlighted from this literature. Indeed, HPV16 load cutoffs are likely influenced by the procedures implemented in these studies, and also by the way to express HPV16 loads. There is a need to standardize HPV16 DNA quantification and the procedures described here could be helpful to achieve these ends. This is especially true in the context of moving from a cytology to a virology based cervical cancer screening policy [40]. Furthermore, because head and neck as well as anal cancers are most if not all associated with HPV16, HPV16 load measurement, either from tumor or liquid biopsy, could probably represent an interesting option in the management of patients [41–45].

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Declarations of interest

None.

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